Studies on the pathogenesis of NSAID-induced damage to the gastrointestinal tract with special reference to the mitochondria.

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STUDIES ON THE PATHOGENESIS OF NSAID-INDUCED DAMAGE TO THE GASTROINTESTINAL TRACT WITH SPECIAL REFERENCE TO THE MITOCHONDRIA

by

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Abstract

Gastrointestinal side-effects pose a major problem for those taking non-steroidal anti-inflammatory drugs (NSAIDs). The mechanism by which NSAIDs adversely affect the gastrointestinal tract is still unclear. The aims of this study were to test the hypothesis suggesting that NSAID enteropathy is a multistage process. Firstly, the effect of NSAIDs on mitochondria was examined, as this is the proposed initial site of damage. Secondly, the "transitional stage" of the pathogenesis (increase in intestinal permeability) was studied, by measuring the transepithelial electrical resistance (TER) of intestinal cells in isolation from any effects of NSAIDs on vascular blood flow.

A range of acidic NSAIDs were found to uncouple oxidative phosphorylation in isolated mitochondria. There was no uncoupling with the non-acidic NSAID nabumetone and also on modification of the acidic side chain (NO-flurbiprofen). The NSAIDs also inhibited mitochondrial electron transport, however, this was not specific to the NSAIDs as the analgesic paracetamol also inhibited. Use of the artificial electron acceptor ferricyanide indicated that electron transport initiated at complex I of the respiratory chain was more susceptible to inhibition by the NSAIDs than that from complex II. However, EPR studies of submitochondrial particles incubated with NSAIDs showed no one complex in particular seemed to be targeted by these drugs. Nevertheless, the EPR work did reveal that the NSAIDs evoked free radical and NO signals, both potentially damaging agents to the gastrointestinal tract.

The TER is inversely proportional to paracellular permeability. Thus, in the present studies, it was found that acidic NSAIDs decreased the TER of the intestinal cell line T84 concomitant with an increase in permeability to the marker ^{51}CrEDTA.

The results strongly indicate that numerous events may interact during NSAID toxicity to the gastrointestinal tract.

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Abbreviations

A	Amps
Å	Angstrom
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANA	Antinuclear antibodies
ANC	Adenine nulceotide carrier
ARA	American Rheumatism Association
Arg	Arginine
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BHM	Beef heart mitochondria
cAMP	Cyclic 3' 5' adenosine monophosphate
CCCP	Carbonyl cyanide *m*-chlorophenylhydrazone
cDNA	Complimentary DNA
^{51}CrEDTA	^{51}Chromium-labelled ethylenediaminetetraacetic acid
CNS	Central nervous system
COX	Cyclooxygenase
DAHP	2,4-Diaminohydroxy pyrimidine
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNP	2,4-Dinitrophenol
EM	Electron microscopy
EPR	Electron paramagnetic resonance
ER	Endoplasmic reticulum
ESR	Electron spin resonance
et al.	And others
FAD	Flavin adenine dinucleotide
$FADH_2$	Reduced flavin adenine dinucleotide
FCCP	Trifluorocarbonylcyanide phenylhydrazone
FDA	Food and Drug Administration
Fe-S	Iron sulphur
FITC	Fluoroscein isothiocyanate
fMLP	*n*-formyl-methionyl-leucyl-phenylalanine
FMN	Flavomononucleotide
g	Spectroscopic splitting factor

γIFN	Gamma interferon
G6P	Glucose-6-phosphate
G6PD	Glucose-6-phosphate dehydrogenase
GFR	Glomerular filtration rate
GI	Gastrointestinal
Glu	Glutamine
GTP	Guanosine triphosphate
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HETE	Hydroxy-5,8,10,14-eicosatetraenoic acid
HMP	Hexose monophosphate
HPETE	12*L*-hydroperoxy-5,8,10,14-eicosatetraenoic acid
I	Current
IC50	Concentration required to give 50% inhibition
IL	Interleukin
IMM	Inner mitochondrial membrane
K	° Kelvin
kDa	Kilodaltons
LT	Leukotriene
μCi	Micro-Curies
MDCK	Madin-Darby canine kidney
6MNA	6-methoxy-2-naphthylacetic acid
Mw	Molecular weight
NADH	Reduced nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced NADP
NSAID	Non-steroidal anti-inflammatory drug
Ω	Ohms
OA	Osteoarthritis
OD	Optical density
PAF	Platelet activating factor
PCA	Perchloric acid
PDA	Patent ductus arteriosus
PFK	Phosphofructokinase
PG	Prostaglandin
P_i	Inorganic phosphate
pKa	Dissociation constant
PMN	Polymorphonuclear leukocytes
P/O	Phosphate/oxygen

PTT	Peak-to-trough
QH_2	Ubiquinol
R	Resistance
RA	Rheumatoid arthritis
RCR	*Respiratory control ratio*
RNA	Ribonucleic acid
SEM	Standard error of the mean
Ser	Serine
SLE	Systemic lupus erythematosus
TCA	Tricarboxylic acid
TER	Transepithelial electrical resistance
TMPD	*N:N:N':N'* tetramethyl p-phenylenediamine
TNF	Tumour necrosis factor
TX	Thromboxane
Tyr	Tyrosine
UQ	*Ubiquinone*
V	Voltage

CHAPTER ONE

INTRODUCTION

Inflammation is a physiological response to injury or irritation of living tissue. It involves many processes including enzyme activation, mediator release, extravasation of fluid, cell migration, tissue breakdown and repair. If, for some reason, this normally rapidly reversible physiological process breaks down and persists, then a pathological one ensues, such as in the case of rheumatic diseases. Anti-inflammatory drugs, as the name suggests, have been developed to inhibit such inflammatory processes by, in the main, inhibiting the generation of pathologically produced prostaglandins. These drugs include the corticosteroids (steroidal) and the non-steroidal antiinflammatory drugs (NSAIDs).

1.1 History of NSAIDs

For thousands of years plants containing salicylates have been used as treatments for various ailments (Gross and Greenberg, 1948). Almost three and a half thousand years ago the application of an extract of the dried leaves of Myrtle to the abdomen and back was suggested by the Ebers papyrus, in order to alleviate rheumatic pains from the womb. A thousand years later Hippocrates discovered that eye diseases could be treated using the juices of the Poplar tree and that both fever and the pain during childbirth could be reduced by chewing Willow leaves.

Such accounts of the medicinal value of Willow leaves, roots, and bark, the Poplar tree, the wintergreen plant, and other sources of salicylates, continued to be documented throughout the Middle Ages and in all parts of the world, with no real idea of the pharmacology of the substance or substances being utilised. The first so-called 'clinical trial' of Willow bark was undertaken by the Reverend Edward Stone in 1763 (Stone, 1763). By administering dried,

ground Willow bark, Stone successfully treated patients suffering from fever and inflammatory disorders.

The work of Stone and others provided the impetus for the isolation and purification of the active principle in Willow bark in order to further study it and put it to clinical use. However, it wasn't until 1829 that the active ingredient in willow bark, a glycoside called salicin, was isolated by Leroux in the pure form exhibiting potent antipyretic activity. Salicin could be hydrolyzed to glucose and salicylic alcohol, and the latter then converted *in vivo* or by chemical manipulation, to salicylic acid. In 1874, salicin was found to lower body temperature and reduce pain and swelling; these being the antipyretic, antiinflammatory and analgesic properties for which salicylates were to be used for a century to come (Maclagan, 1876). In the same year (1874), the practical synthesis of salicylic acid had been elucidated by Kolbe and Lautemann (Friend, 1974). Salicylic acid then soon had its first use in the treatment of rheumatoid fever (Stricker, 1876).

Shortly after establishing the therapeutic effectiveness of salicin and salicylic acid in rheumatic fever, sodium salicylate became the preferred compound because of its greater water solubility, lower toxicity and the fact that it was relatively easy to manufacture. Sodium salicylate was effective in the treatment of chronic rheumatoid arthritis, fever and gout. However, the sharp bitter taste of sodium salicylate plus its frequent side effects of gastric irritation, meant that the compound could not be prescribed for a long period of time.

In 1897, Hoffman acetylated the hydroxyl group on the benzene ring of salicylic acid to produce acetylsalicylic acid (figure 1.1). His colleague Dreser, having carried out detailed pharmacological and toxicological tests, reported

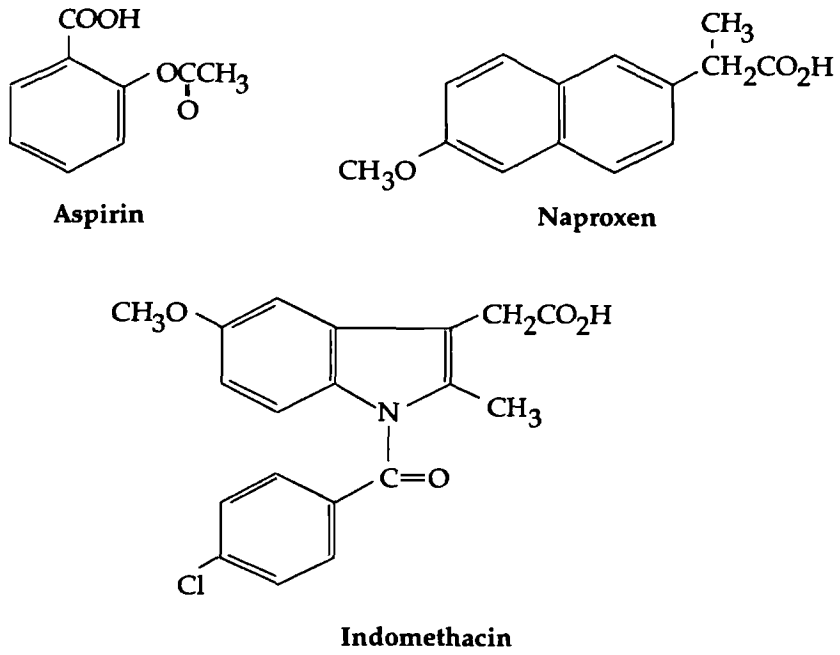

Figure 1.1 Structures of NSAIDs

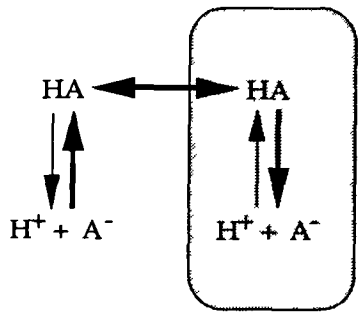

Figure 1.2 Ion trapping of weak acids in an acidic environment
NSAIDs being weak acids remain unionised (HA) in the acidic environment of the stomach (left). Therefore, the concentration of HA is greater here than inside the gastric mucosal cell (right). But in order to maintain an equilibrium across the cell membrane HA passes into the cell, facilitated by the high lipophilicity of the drugs. Then the higher intracellular pH means that the NSAIDs become ionised ($H^+ + A^-$) and trapped within the cell. Modified from Brooks and Day (1991).

that the new compound was readily converted to salicylic acid upon absorption into the body plus had a more pleasant taste and was better tolerated by the gastric mucosa (Dreser, 1899). He thus introduced acetylsalicylic acid onto the market under the name of 'aspirin' (*a* for acetyl, *spir* for the genus Spirae, and *-in* as a popular suffix for drugs of the times). By the early 1900's the main therapeutic actions of aspirin were known; antipyretic, antiinflammatory and analgesic.

However, the search for a better tolerated and more effective antiinflammatory agent continued when, in the late 1940's, corticosteroids were found to relieve the symptoms of rheumatoid arthritis (Hench *et al.*, 1949). This set in motion intensive research during the 1950's, producing many synthetic corticosteroid analogues with increasing potency. However, even at therapeutic doses, side-effects resulted, due to the administration of grossly unphysiological quantities of the hormone preparation. The side-effects included a clinical picture similar to that of Cushing's syndrome, osteoporosis, diabetes mellitus, reduced resistance to infections and hypertension.

This therefore stimulated research into anti-inflammatory drugs of the non-steroidal type in the late 1950's, with a view to dissociating the serious side-effects of corticosteroids from their antiinflammatory activities. The first non-steroidal anti-inflammatory drug, phenylbutazone, was brought onto the market in 1953 but again increasing reports of adverse effects soon followed (Smyth, 1982).

Indole derivatives of the potent anti-inflammatory mediator serotonin were prepared, to see if they would in some way affect serotonin metabolism (Shen *et al.*, 1963). This study culminated with the discovery of indomethacin in

1961, which was able to mimic the antiinflammatory activity of corticosteroids without their serious systemic side effects (figure 1.1). Indomethacin was shown to have both antiinflammatory and analgesic effects in rheumatoid and acute gouty arthritis, and was also highly effective in the treatment of ankylosing spondylitis and moderate osteoarthritis (Hart and Boardman, 1963).

The discovery and clinical usage of indomethacin prompted further research into nonsteroidal compounds. Independent studies at the Boots Laboratories (England) resulted in the development of a propionic acid derivative ibuprofen, another widely used NSAID, which was brought onto the market in 1969 (Boardman *et al.*, 1970). Later its derivative flurbiprofen was successfully developed. Minor chemical modifications to the propionic acid structure gave rise to large range of NSAIDs more potent than indomethacin (Shen, 1972) and with apparently less gastrointestinal side-effects (Shen, 1984), such as fenbufen and naproxen (figure 1.1). However, it has since been shown that this increase in tolerability requires further clarification.

Despite the emergence of numerous NSAIDs, their mode of action was not known until 1971, when it was reported that aspirin and indomethacin could inhibit prostaglandin formation at therapeutic concentrations (Smith and Willis, 1971; and Vane, 1971). As prostaglandins were shown to modulate important cellular and vascular changes in inflammation, the inhibition of their biosynthesis would therefore suppress inflammation, pain and fever. These findings prompted the development of yet more compounds, such as the fenamates and diflunisal. Analogues of fenamates include diclofenac, another potent NSAID (Ku *et al.*, 1975). Diflunisal, on the other hand, is a new analogue of salicylic acid, approximately four times more potent than aspirin as an analgesic-antiinflammatory agent, with a longer duration of

action, plus is less irritating to the stomach than most NSAIDs (Stone *et al.*, 1977).

However, the gastrointestinal and CNS side effects (dizziness, headache) seen with NSAIDs meant that there was still room for improvement. Almost a decade after the advent of indomethacin, sulindac, an analogue of indomethacin was established as a new type of prodrug (Shen *et al.*, 1972). Sulindac is a relatively inert compound, but in the body it is converted to a biologically active metabolite, which is comparable to indomethacin at inhibiting prostaglandin biosynthesis (Duggan *et al.*, 1977). The prodrug concept was tested in short-term endoscopy trials and found to be well tolerated (Lanza, 1989). Table 1.1 compares the incidence of damage caused by conventional NSAIDs with that of proNSAIDs. However, short-term tolerability is not predictive of long-term safety, thus sulindac and subsequent pro-NSAIDs have been found to be toxic in the long-term. A nonacidic prodrug which has emerged is nabumetone which is oxidized *in vivo* to an active metabolite, and has been shown to be relatively safe (Boyle *et al.*, 1982). Table 1.2 summarizes the evolution of NSAIDs.

1.2 Uses of NSAIDs

NSAIDs are one of the most frequently used drugs both in the U.K. and the rest of the world. For example, over 100 million prescriptions were written worldwide in 1989, equivalent to 5% of the population in most developed countries. More than 20% of patients admitted to hospital in the United Kingdom and Australia are taking NSAIDs. At any one time there are over 50 NSAIDs marketed or on trial throughout the world, though most countries have between 7 and 17 available for use.

Table 1.1 **Incidence of gastric ulcer in normal subjects taking NSAIDs and pro-NSAIDs**

Drug	Dose (mg/day)	% Ulcer Incidence	pK_a
Aspirin	3900	9.8	3.5
Ibuprofen	1600	9.1	5.2
Naproxen	750	8.8	4.2
Indomethacin	150	2.9	4.6
Piroxicam	20	4.0	5.3
Sulindac†	400	2.6	4.5
Fenbufen†	1000	0.0	4.3

† = Prodrugs. The most commonly used doses are shown. Adapted from Lanza (1989).

Table 1.2 **The emergence of antiinflammatory compounds**

Major Research	Decade
Herbal plants e.g. *Salix, spiraea*	before 1760
Aspirin and salicylates	1900
Pyrazolones, phenylbutazone	1940
Corticosteroids	1950
Indomethacin, ibuprofen, fenamates (NSAIDs)	1960
Proliferation of NSAIDs	1970

Modified from Shen (1984).

There follows a brief description of the disorders that NSAIDs are most frequently prescribed for.

1.2.1 Musculoskeletal Disorders

The main clinical application of NSAIDs relies on their analgesic and antiinflammatory properties to treat the signs and symptoms musculoskeletal disorders, with more than 20 million prescriptions written each year in the U.K. (Wright, 1988). Over one third of elderly bed-ridden or house-bound patients suffer from severe rheumatic disorders requiring NSAIDs. Fifty percent of NSAID use is for osteoarthritis (OA), explaining the rise in NSAID use in the elderly, and about 20% of NSAIDs are taken for back pain. The widespread popularity of these drugs means that some are now available over the counter without prescription. However, NSAIDs provide only symptomatic relief from the pain and inflammation associated with the disease and do not prevent the progress of pathological injury to the tissues in the long term. Table 1.3 summarizes the main musculoskeletal disorders treated by NSAIDs.

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory disorder affecting approximately 1% of the world's population, but whose aetiology is not known (Hochberg, 1981). It is more common in women and can occur at any age, although the incidence appears to increase with age. Table 1.4 shows the clinical features of rheumatoid arthritis.

The treatment of RA generally begins with the symptomatic treatment of the inflammation with NSAIDs in addition to rest and corticosteroid injections. NSAIDs reduce, but do not completely eliminate, the signs and symptoms of

Table 1.3 Main musculoskeletal disorders treated by NSAIDs

Type of Disorder	Examples
Inflammatory synovitis	Rheumatoid arthritis
	Gout
	Pseudogout
	Septic arthritis
Degenerative arthritis	Osteoarthritis
Vasculitis & connective tissue diseases	Systemic lupus erythematosus
	Polymyalgia rheumatica
	Temporal arteritis
Soft tissue rheumatism	Rotar cuff shoulder lesions
	Frozen shoulder
Back pain	Mechanical back pain
	Lumbar spondylosis & spondylolisthesis
	Spinal stenosis
	Osteoporotic vertebral fracture
	Spinal malignancy

Table 1.4 Clinical features of rheumatoid arthritis

Clinical Features
1. Morning stiffness in and around joints
2. At least 3 joint areas have simultaneous swelling or fluid in the soft tissue
3. Swollen wrist
4. Subcutaneous nodules over bony prominences
5. Abnormal amounts of "rheumatoid factor" in the serum
6. Involvement of joint areas on both sides of the body at the same time
7. Erosions or decalcification of bone

A patient with at least 4 of the 7 features is classified as having rheumatoid arthritis (revised ARA criteria, 1988).

inflammation so there is no obvious long-term protective benefit with these drugs. In addition to their antiinflammatory effect, NSAIDs decrease pain, fever and platelet adhesiveness (Borigini and Paulus, 1995).

Gout

Gout is a syndrome caused by an abnormality in uric acid metabolism resulting in the deposition of sodium urate crystals in (i) the joints, causing acute gouty arthritis, (ii) the soft tissue, causing tophi (hardened masses) and tenosynovitis (inflammation of tendons) or (iii) the urinary tract, causing urate stones. The biochemical abnormality is hyperuricaemia resulting from an overproduction or decreased excretion of uric acid. Although colchicine is often used to treat acute attacks of gout, the potent NSAIDs provide an excellent alternative by bringing about short-term pain relief. Except in the elderly, indomethacin, aspirin and diflunisal, appear very effective in the acute phase because of their analgesic and uricosuric properties, bringing substantial relief within 24-48 hours. Diflunisal has been suggested as the preferred drug as lower doses are required plus it causes less gastrointestinal irritation, though this claim needs to be substantiated (Axon and Huskisson, 1992).

Osteoarthritis

Osteoarthritis (OA) is the commonest type of arthritis, found in 20% of the population as a whole and in more than 75% of people over the age of 70 (WGPO, 1966). Osteoarthritis is the slow degeneration of cartilage characterized by the gradual development of joint pain, stiffness, and limitation of motion. Osteoarthritis is particularly problematic in the large weight-bearing joints i.e. knee and hip; the commonest cause of disability in the elderly (Doherty, 1992). NSAIDs are extensive y used in OA since at low doses they are effective analgesics and at higher doses have antiinflammatory

activity, beneficial for the treatment of the inflammatory synovitis that often results from the local degenerative damage (Gramas and Lane, 1995).

Polymyalgia Rheumatica

Patients with polymyalgia rheumatica have widespread joint and muscle stiffness, and, although quite disabling, complete recovery occurs in most patients within 1 to 2 years (Gardner, 1995). Salicylates were the first drugs used to treat polymyalgia rheumatica, giving partial to complete relief of the musculoskeletal symptoms (Barber, 1957). Other NSAIDs have been used with similar effects, allowing lower doses of corticosteroids to be used and thus avoiding the osteoporotic side-effects.

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is the commonest connective tissue disorder, characterized by the presence of antinuclear antibodies (ANA) in the serum. The disease affects numerous organs and systems in the body (joints, skin, lungs, heart, kidneys, nervous system, eyes, gastrointestinal system), but arthralgia and rashes are the clinical features most often seen, with cerebral and renal disease being the most serious associations. NSAIDs are effective for the frequent arthralgic symptoms and the fever, but not for the accompanying malaise and lethargy. Aspirin and other NSAIDs have been shown to be highly effective in alleviating the fever, inflammation and serositis (inflammation of serous membrane) of SLE (Hughes, 1979).

1.2.2 Non-Rheumatological Uses

Dysmenorrhoea

NSAIDs are also efficacious in controlling the painful symptoms of dysmenorrhoea (Furniss, 1982). Women suffering from dysmenorrhoea

normally have painful abdominal cramps together with various other symptoms including fever, headache, nausea, vomiting, diarrhoea, breast tenderness, nervousness and mood swings. The pathogenesis of dysmenorrhoea appears to be related to increased production and release of endometrial prostaglandins during menstruation, which results in increased and abnormal uterine activity (Dawood, 1981). Thus, by suppressing prostaglandin production, NSAIDs can control the action of prostaglandins on the endometrium and uterus, thereby reducing menstrual flow, cramping, headache, oedema, bloating, breast tenderness and mood changes. NSAIDs, in particular indomethacin, naproxen sodium and the fenamic acids, are the treatment of choice for dysmenorrhoea (Evens, 1984); aspirin, on the other hand, has been found to be relatively ineffective (Marx, 1979). Table 1.5 gives a summary of the clinical efficacy of NSAIDs in dysmenorrhoea.

Patent Ductus Arteriosus

NSAIDs have also been found to be useful in the treatment of patent ductus arteriosus (PDA) (Evens, 1984). A PDA is an anatomical structure in the foetus *in utero*, that enables communication between the main pulmonary artery and the aorta, allowing blood to be shunted away from the pulmonary vasculature. If the PDA fails to close at birth, cardiovascular abnormalities result, such as heart murmur and tachycardia. Prostaglandins play a role in maintaining the patency of a ductus arteriosus during foetal life and NSAIDs, in particular indomethacin, are considered appropriate drug therapy, along with fluid restriction, and/or diuretic therapy, and/or ventilatory support, to close a PDA.

General Analgesia

Although NSAIDs were originally introduced onto the market to treat the pain and inflammation associated with chronic inflammatory conditions,

Table 1.5 The clinical efficacy of NSAIDs in dysmenorrhoea

NSAID	Clinical Relief
Ibuprofen	66-100%
Mefenamic acid	93%
Ketoprofen	90%
Naproxen sodium	78-90%
Indomethacin	73-90%
Tolfenamic acid	88%
Flufenamic acid	77-82%
Suprofen	60-80%
Piroxicam	Effective
Aspirin	Ineffective

Modified from Dawood (1988).

Table 1.6 NSAIDs used as analgesics

Indicated as general purpose analgesic	Indicated for painful shoulder (bursitis/tendinitis)
Aspirin and other salicylates	Phenylbutazone
Acetaminophen	Oxyphenbutazone
Mefenamic acid	Indomethacin
Ibuprofen	Sulindac
Naproxen	
Naproxen sodium	
Fenoprofen calcium	
Ketoprofen	
Diflunisal	

Source: Beaver (1988).

now many of these drugs are used as general analgesics. When employed as analgesics, however, these drugs are only effective against pain of low-to-moderate intensity (Insel, 1996). Though their maximal effects are much lower than narcotics, NSAIDs have the added advantage of not being associated with unwanted effects on the central nervous system (CNS) such as respiratory depression, physical dependence or tolerance, thus are particularly beneficial when used in the treatment of recurrent or chronic painful conditions (McCaffery, 1985). Table 1.6 lists the NSAIDs commonly used as analgesics.

Indomethacin has been shown to prevent or relieve the pain of migraine, and naproxen, mefenamic acid and fenoprofen, as well as indomethacin, appear to be very effective in the treatment of menstrual-related migraines. Research has shown that NSAIDs such as ibuprofen and aspirin can alleviate pain following postpartum episiotomy, dental extractions, herniorrhaphy and orthopaedic surgery (Dionne and Cooper, 1978; and Slavic-Svircev, 1984). Although highly controversial, NSAIDs have been found to very effective in reducing cancer pain especially in bone metastasis and may actually inhibit the progress of bone tumours (Ventafridda, 1980).

Antipyretic Use

NSAIDs can bring down a raised body temperature except when elevated by such factors as exercise or increased ambient temperature (Insel, 1996). Indomethacin has been shown to give remarkable clinical improvements by quickly and completely diminishing the fever associated with Hodgkin's disease and other lymphoid malignancies, where other antipyretic agents have failed (Berbetto *et al.*, 1993).

Cardiovascular Diseases

Low doses of aspirin have a potent and prolonged duration of action on platelets, and therefore the drug is used in the treatment or prophylaxis of diseases associated with platelet hyperaggregability, such coronary artery disease and deep-vein thrombosis (Patrono, 1994). The antithrombotic action of aspirin is utilised to prevent the clinical complications of atherosclerosis (Schror, 1997).

Pregnancy

Aspirin is administered to pregnant women who have a high risk of developing hypertension in order to lower the blood pressure and prevent preeclampsia (Imperiale and Petrulis, 1991). Indomethacin and aspirin can be used as to suppress uterine contractions in women with preterm labour (Mosler, 1975).

1.3 Chemical Classification of NSAIDs

NSAIDs come from a variety of chemical classes (table 1.7). NSAIDs are primarily organic acids, with the exception of nabumetone.

1.4 Pharmacokinetics of NSAIDs

Pharmacokinetics describes the disposition of a drug and its metabolites in the body with relation to time. It has been suggested that the pharmacokinetics of NSAIDs contributes not only to their therapeutic effects but also to the type and incidence of their side-effects (Brune and Lanz, 1985). The main processes which affect the disposition of a drug, or its concentration in the blood, are:

Table 1.7 Chemical classification of nonsteroidal antiinflammatory drugs

Chemical Family	NSAIDs
Carboxylic acid — acetylated	Aspirin
— nonacetylated	Diflunisal, magnesium salicylate, salicylamide, salsalate, sodium salicylate
Acetic acid	Diclofenac, indomethacin, tolmetin, sulindac, etodolac
Propionic acid	Ibuprofen, naproxen, fenoprofen, ketoprofen, fenbufen, flurbiprofen
Fenamic acid	Flufenamic acid, mefenamic acid
Enolic acid	Piroxicam, tenoxicam, phenylbutazone, oxyphenbutazone
Nonacidic	Nabumetone

From Brooks and Day (1991).

absorption, protein binding, its distribution within the body, metabolism and excretion.

1.4.1 Absorption

All NSAIDs are rapidly and almost completely absorbed intact following oral administration, with the exception of aspirin (and also diclofenac, tolfenamic acid and perhaps fenbufen), which undergoes presystemic hydrolysis to form salicylic acid before being absorbed (Verbeeck *et al.*, 1983). Absorption takes place in the stomach and upper small intestine by passive diffusion and is affected by the pH of the aqueous layer near the mucous membrane (Brogden, 1986). The majority of NSAIDs are weak acids, with ionisation constants (pK_a's) ranging from 2.5 to 4.7, thus become unionised in the acidic environment of the stomach. Under these conditions, NSAIDs are lipid soluble and freely partition across the lipid bilayer membrane of gastric lining cells. On the one hand, the ease with which NSAIDs are absorbed within the stomach may lead to a rapid build-up of effective plasma concentrations, causing early analgesic effect (Brune and Lanz, 1985). On the other hand however, as the intracellular pH is higher, the drug becomes ionised and subsequently "ion-trapped" within the mucosal cells (figure 1.2). Such high local drug concentrations, together with prostaglandin synthesis inhibition, could contribute to the gastrointestinal side-effects seen with NSAIDs (Halter, 1988).

Although absorption of the NSAID may be delayed by taking antacids (Verbreeck *et al.*, 1979) or food prior to the drug, the amount of drug

eventually absorbed is the same. However, under these circumstances in the small intestine, the fraction of the NSAID dose absorbed is larger (Mortensen and Rennebohm, 1989). Nevertheless, slow absorption is not a problem when these drugs are used to treat chronic rheumatic diseases, though rapid absorption may be desirable when NSAIDs are being employed as analgesics or antipyretics. Enteric coated tablets of e.g. aspirin and diclofenac, are often used to improve gastrointestinal tolerability. This has the effect of delaying release and absorption of the drug until within the small intestine, with the aim to reduce gastric damage by bypassing the "topical" effect of the drug at this site (Day *et al.*, 1988), though this does not reduce long-term toxicity.

1.4.2 Distribution

Following its administration and absorption, a drug is then distributed throughout the body. NSAIDs bind substantially to serum albumin (90-99%). Therefore, in most cases, less than 1% of the total plasma concentration is in the unbound form of the drug and available to distribute in extravascular tissues (Verbeeck *et al.*, 1983). This would explain why the apparent volumes of distribution of NSAIDs are very low, usually less than 0.2L/kg.

The weak acidity of NSAIDs means that they are completely ionised at plasma pH and, as already mentioned, only unionised molecules are lipid soluble and able to diffuse through biological membranes (Brune and Lanz, 1985). Therefore this property of the NSAIDs, together with the fact that they are protein bound, means that leakage out of the plasma space is slow in most parts of the body except in inflamed tissues, liver, bone marrow and spleen.

1.4.3 Half-Life

The half-life ($t_{1/2}$) of elimination of a drug is the time take for a given concentration of drug to fall by half. The interval between doses is normally set at approximately the half-life in order to limit plasma fluctuations of the drug. Furthermore, it generally takes 3-5 half-lives for the drug to reach steady-state concentrations.

NSAIDs can be divided into two categories on the basis of their half-lives, those with half-lives less than 6h and those with half-lives greater than 12h. This distinction has been made in order to serve as a rough guide to the dosage regimens of these drugs. It was also used from a promotional standpoint, rather than a factual one, to suggest better tolerability.

NSAIDs With Long Half-Lives

NSAIDs with long half-lives of elimination are usually given one to two times a day, thus the plasma concentration and the pharmacological effect remain relatively constant throughout the day. As the half-life of elimination is equal to the half-life of accumulation, drugs with long half-lives accumulate in the body slowly, albeit to a considerable degree, and for a long time after the start of dosing. During long-term treatment, NSAIDs with long half-lives are generally administered as conventional tablets containing the acid forms of the drug, as modifying the rate of absorption has little effect on the day-to-day plasma concentration of the drug. Table 1.8 shows a list of NSAIDs with long half-lives.

NSAIDs With Short Half-Lives

NSAIDs with short half-lives are usually administered every 6-8h, which is considerably longer than their half-lives and so plasma concentrations

Table 1.8 Half-lives of NSAIDs

NSAID	Half-life (h)
Short half-lives	
Aspirin	0.25
Flurbiprofen	3.8
Ibuprofen	2.1
Diclofenac	1.1
Indomethacin	4.6
Tolmetin	1
Etodolac	3
Flufenamic acid	1.4
Long half-lives	
Salicylate	2-15
Diflunisal	13
Fenbufen*	11
Naproxen	14
Sulindac*	14
Nabumetone*	26
Piroxicam	57
Azapropazone	15
Phenylbutazone	68

* Active metabolite. Taken from Day *et al.* (1988).

fluctuate significantly during a dosage interval. Despite the swinging plasma concentrations, analgesic and anti-inflammatory activity is well maintained with these NSAIDs (Graham *et al*, 1984). In contrast to NSAIDs with long half-lives, the plasma concentration of those with short half-lives is very much affected by the rate of absorption. Slowing the rate of absorption markedly decreases the peak plasma concentrations of these drugs. Sustained release preparations of several NSAIDs with short half-lives have been produced in order to maintain active plasma levels for a prolonged period of time, although there appears to be no improvement in therapeutic response (Yeh, 1985). Table 1.8 lists NSAIDs with short half-lives.

1.4.4 Synovial Kinetics

In inflammatory joint diseases, the main site of action of NSAIDs is most likely to be in the synovial compartment. Since passage of the NSAIDs across the synovial membrane occurs by simple diffusion, it is determined by protein binding of the drug plus other physicochemical properties such as pK_a, lipophilicity, molecular size and distance between the plasma and the synovial compartment (i.e. the amount of oedema and inflammation in the tissues) (Furst, 1994). In rheumatoid arthritis, the synovial blood flow is diminished plus there is an accumulation of metabolic waste products (Verbreeck, 1988). There is also a reduction in serum albumin levels which means higher unbound drug concentrations, and it is only the unbound drug which is pharmacologically effective. In addition, there is a decrease in pH of the synovial fluid, thus NSAIDs become "ion-trapped" within the synovial cells. Therefore, the selective concentration of NSAIDs in the cells of the synovium may be responsible for some of the characteristic therapeutic and adverse effects of NSAIDs.

1.4.5 Clearance

Clearance describes all the processes leading to the removal of a drug. At a fixed dose of drug, a lower than average clearance leads to a higher than average level in all tissues including plasma. Generally, total body clearance of NSAIDs is relatively low (Verbeeck *et al.*, 1983).

Hepatic Clearance

NSAIDs are mainly cleared by hepatic metabolism and the metabolites are generally inactive. In man most of the NSAIDs are metabolised by cytochrome P450-mediated oxidation and/or glucuronide conjugation (table 1.9). Hepatic microsomal oxidation is affected by several factors including advancing age, hepatocellular disease (e.g. cirrhosis), enzyme inducers or inhibitors, and genetic constitution of the patient (Vesell, 1982). Glucuronide conjugation, on the other hand, appears to be less susceptible to such interfering factors (Verbreeck, 1988).

Hepatic clearance of NSAIDs is independent of liver blood flow but dependent on protein binding. If an NSAID is displaced from its binding site, there is an increase in clearance and a decrease in the total plasma concentrations of the drug. Metabolism of the NSAIDs vary markedly between patients, resulting in huge variations in the steady-state concentrations of the drugs.

Metabolic activation

Most NSAIDs are metabolised to an inactive metabolite which is then excreted in the urine. Alternatively, there are also compounds which are inactive themselves but have active metabolites. The best known example is aspirin, which although itself can inhibit prostanoid synthesis, owes its

Table 1.9 Hepatic metabolic pathways of NSAIDs

NSAID	Primary Metabolic Pathway(s)	Active Metabolites
Aspirin	Hydrolysis	Salicylic acid
Salicylic acid	Conjugation (A,G), oxidation	
Diflunisal	Conjugation (G,S)	Oxyphenbutazone
Phenylbutazone	Oxidation	
Azapropazone	Oxidation	
Indomethacin	Oxidation, conjugation (G)	
Sulindac	Reduction, oxidation	Sulindac sulphide
Tolmetin	Decarboxylation, conjugation (G)	
Ibuprofen	Oxidation	
Naproxen	Conjugation (G), oxidation	
Ketoprofen	Conjugation (G)	
Fenoprofen	Conjugation (G)	
Carprofen	Conjugation (G)	
Fenbufen	Oxidation	γ-OH-4-biphenyl butyric acid, 4-biphenyl acetic acid
Flurbiprofen	Oxidation	
Benoxaprofen	Conjugation (G)	
Pirprofen	Oxidation	
Tiaprofenic acid	Conjugation (G), reduction/oxidation	
Oxaprozin	Oxidation, conjugation (G)	Hydoxyoxaprozin
Diclofenac	Oxidation	3-OH- diclofenac, 4-OH-diclofenac
Aclofenac	Conjugation (G)	4-OH-3-chloro-phenyl acetic acid
Tolfenamic acid	Oxidation	
Piroxicam	Oxidation	
Isoxicam	Oxidation	

A = amino acid conjugation; G = glucuronidation; S = sulphation. Adapted from Verbreeck (1988).

pharmacological properties to its metabolite salicylate, which is an active anti-inflammatory agent. Salicylate, however, is eliminated more slowly than aspirin, and the accumulation of salicylate puts a limit on how much aspirin can be administered.

Until metabolised, the NSAIDs nabumetone, fenbufen and sulindac, have little intrinsic activity with respect to cyclooxygenase inhibition. Such drugs are known as 'prodrugs'. The drug sulindac is oxidised to the sulphone then reversibly reduced in the large intestine by microflora to the sulphide (figure 1.3a); the latter metabolite is responsible for the anti-inflammatory activity of parent compound (Strong *et al.*, 1985). The metabolism of sulindac is reversible and in the kidney the sulphide is converted back to the inactive sulindac, a possible explanation for the reduced effect of sulindac on glomerular filtration when compared to other NSAIDs (Sedor *et al.*, 1984). Fenbufen is converted by β-oxidation to the active metabolites biphenylacetic acid and γ-hydroxybiphenylbutanoic acid (figure 1.3b), which are responsible for its antiinflammatory and analgesic activity (Child *et al.*, 1977), but it is only biphenylacetic acid which inhibits prostaglandin synthesis (Sloboda *et al.*, 1980). The conversion of nabumetone to its active metabolite 6-methoxy-2-naphthylacetic acid (6-MNA), occurs in the liver by oxidative cleavage of the side chain to the acetic acid derivative (figure 1.3c). Final elimination in the urine follows glucuronide or sulphide conjugation of the metabolites (Friedel *et al.*, 1993).

Liver disease

Little is known about the pharmacokinetics of NSAIDs in liver disease. However, it has been shown that the plasma levels of sulindac sulphide increase four-fold in alcoholic liver disease (Juhl *et al.*, 1983) and clearance of azapropazone decreases as liver function deteriorates (Breuing *et al.*, 1981).

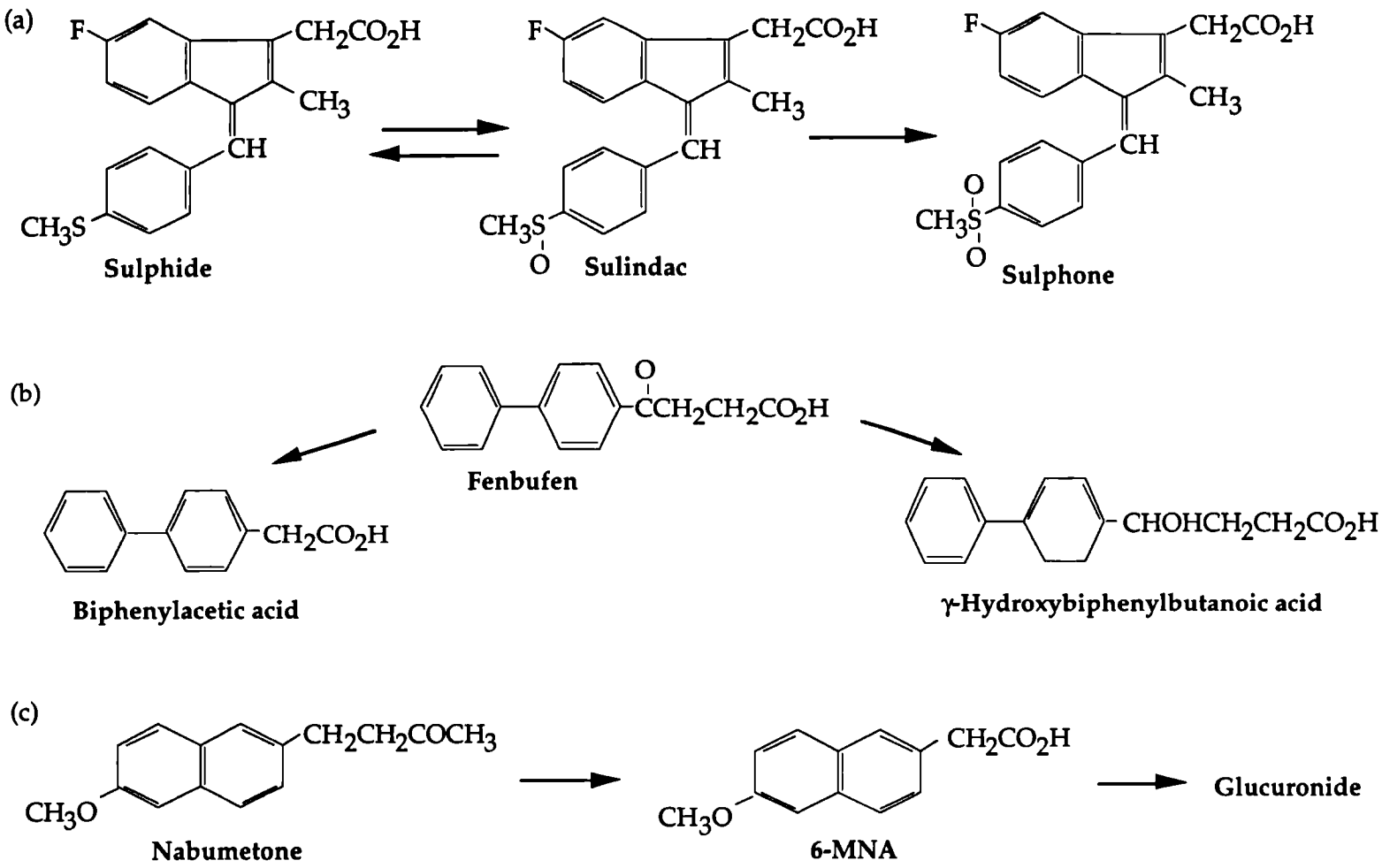

Figure 1.3 **Metabolism of prodrugs**
(a) Sulindac, (b) fenbufen and (c) nabumetone

Renal Clearance

In most cases, renal excretion of intact NSAID accounts for only a small proportion (<10%) of the administered NSAID. Moreover, being mainly weak acids, the renal clearance of NSAIDs increases with increasing urinary pH. However, overall the renal clearance of NSAIDs is much lower than their hepatic clearance even when the urinary pH is high. Thus, changes in urinary pH do not significantly affect the total body clearance of NSAIDs. The main exception is salicylate which, when urinary pH exceeds 6.5, has increased renal clearance and decreased plasma concentration (Levy and Leonards, 1971), a feature that is utilised for the treatment of aspirin overdose.

Renal failure

Although ordinarily only small amounts of the given doses of the NSAIDs are excreted unchanged, in renal failure the clearance of several NSAIDs including indomethacin, diflunisal, ketoprofen, fenoprofen, naproxen is decreased. This is due to the retention of the acyl glucuronide metabolites of the NSAIDs in subjects with renal failure which are then hydrolysed back to the parent compound *in vivo* (Meffin, 1985). Thus in patients with renal failure, it would be preferable to use NSAIDs which are not metabolised directly to glucuronides.

Biliary Clearance

The metabolites of most NSAIDs are also excreted to a significant extent via the bile, for example indomethacin (Kwan *et al.*, 1975), sulindac (Dujovne *et al.*, 1983), tolfenamic acid (Pentikainen *et al.*, 1984), carprofen and piroxicam (Verbreeck, 1988). Biliary excretion of glucuronide conjugates may be followed by their hydrolysis in the gut reexposing the intestine to the parent compound with subsequent reabsorption. This enterohepatic cycle could be important in

prolonging the duration of action of the drug, as well as contributing to their gastrointestinal toxicity. 6-MNA is not excreted in the bile, perhaps another reason why its precursor nabumetone appears to cause less gastrointestinal injury.

1.5 Mechanism of Action of NSAIDs

The beneficial effects of NSAIDs are primarily due to their ability to inhibit prostaglandin synthesis (Vane, 1971). In order to understand the mechanism by which NSAIDs act, it is important to have a clear picture of prostaglandin biosynthesis.

1.5.1 Prostaglandin Biosynthesis

Prostaglandins were first isolated over 60 years ago from ram seminal vesicles (von Euler, 1937); their name 'prostaglandin' was a reference to the source of origin the prostate gland. It is now known that the enzymes used to synthesize prostaglandins are ubiquitous and present in virtually all mammalian tissues, with the exception of red blood cells (Smith, 1987).

The precursor of prostaglandins is a C-20 fatty acid 5,8,11,14-eicosatetraenoic acid, more commonly known as arachidonic acid (figure 1.4). Arachidonic acid is converted to prostaglandins and thromboxanes by the enzyme cyclooxygenase and to leukotrienes by lipoxygenase. Prostaglandins, leukotrienes and thromboxanes are a group of oxygenated lipids collectively known as 'eicosanoids'. Eicosanoids are local hormones being short-lived and, as they are not stored within cells, must be synthesized *de novo* from arachidonic acid which is produced upon mechanical or physiological

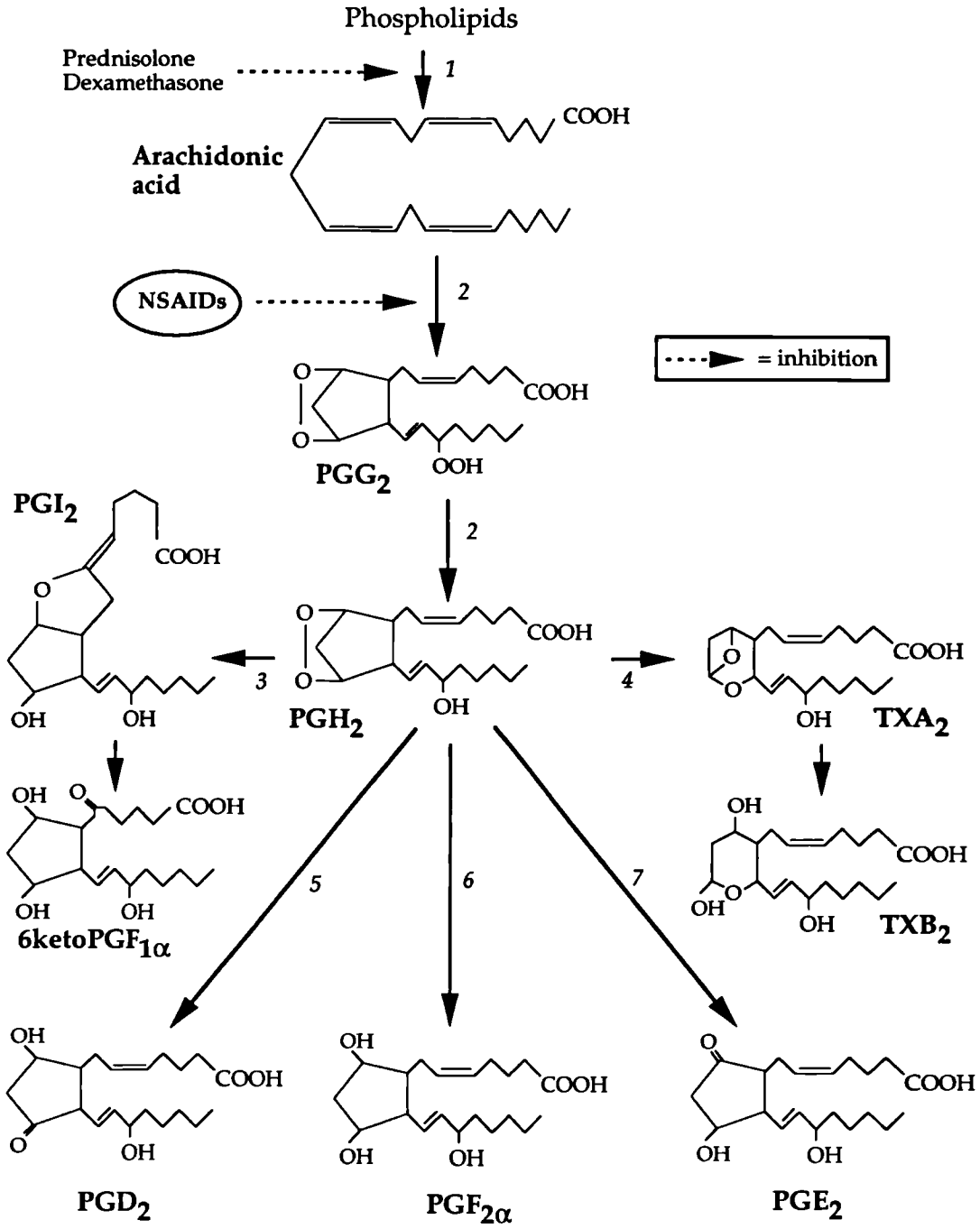

Figure 1.4 The arachidonic acid cascade

This diagram illustrates prostaglandin biosynthesis (see main text).

1 = phospholipases; 2 = COX-1 or COX-2; 3 = PGH synthase; 4 = TXA synthase; 5 = PGD synthase; 6 = PGF synthase; and 7 = PGE isomerase.

stimulation of the cell. The eicosanoids act near or at their sites of synthesis, with the nature of these effects varying from one type of cell to another. Arachidonic acid is not present in cells in the free form but is esterified in lipids and phospholipids and so needs to be released by a hydrolase enzyme.. As arachidonic acid is most commonly esterified to cellular phospholipids, it is the enzyme phopholipase A_2 which is normally responsible for its cleavage. Thus, prostaglandin biosynthesis is thought to be regulated by the level of arachidonic acid mobilization and hence substrate availability.

Although prostaglandins are all 20-carbon carboxylic acids containing a five carbon ring, there are differences in the number of double bonds and whether the cyclopentane portion is a diol or a keto alcohol (Fessenden and Fessenden, 1986). For example, in conventional terminology, PG means prostaglandin, E means the keto alcohol, and F means the diol. The subscript number denotes the number of carbon-carbon double bonds present outside the ring and the subscript α refers to the configuration of the hydroxyl group at C-9 (*cis* to the carboxyl side chain).

As shown in figure 1.4, the initial step of prostaglandin synthesis is the removal of hydrogen at C-13 of arachidonic acid and peroxidation at C-11, followed by the addition of oxygen at C-15 and ring closure between C-8 and C-12 to form the unstable cyclic endoperoxides PGG_2 and PGH_2. This reaction is catalyzed by cyclooxygenase. Alternatively, removal of the C-13 hydrogen from arachidonic acid can also facilitate peroxidation at C-15 in which case 15-HETE (15-hydroxyeicosatetraenoic acid) results via its corresponding hydroperoxy acid. In addition, if there is no cyclisation of arachidonic acid then 11-HETE is produced.

The cyclic endoperoxides are converted to various products depending upon the tissue in which they are manufactured. The eicosanoid thromboxane A_2 (TXA_2), is synthesized in a number of tissues including platelets (Needleman *et al.*, 1976) and polymorphonuclear leukocytes (Higgs *et al.*, 1976a), and has potent platelet aggregating activity. Prostacyclin (PGI_2) is the major cyclooxygenase product of blood vessel walls and can prevent platelet aggregation, relax smooth muscle and is also a potent vasodilator (Moncada and Vane, 1977). TXA_2 and prostacyclin then break down to relatively stable and inert products, thromboxane B_2 and 6-oxo-$PGF_{1\alpha}$ respectively.

The other stable prostaglandins identified so far PGD_2, $PGF_{2\alpha}$ and PGE_2 are relatively minor products of fatty acid oxidation. However, prostaglandin E_2 (PGE_2) is the main eicosanoid detected in inflammatory lesions, causing the vasodilatation and erythema (redness) characteristic of acute inflammation (Higgs and Salmon, 1979). PGE_2 is also responsible for the fever associated with bacterial and viral infections (Saxena *et al.*, 1979). Furthermore, although PGE_2 itself does not directly cause pain, it acts along with other mediators, sensitizing pain receptors on afferent nerve endings to the actions of bradykinin and histamine (Moncada *et al.*, 1979).

1.5.2 The Cyclooxygenase Enzyme

The enzyme cyclooxygenase (COX) catalyses the first step in the biosynthesis of prostaglandins, converting arachidonic acid to PGH_2, which is then rapidly converted to one of several prostanoids. This enzyme is bifunctional: the initial cyclooxygenase reaction, the target for NSAIDs, converts arachidonic acid to PGG_2, while the subsequent peroxidase reaction converts PGG_2 to PGH_2.

Two isoforms of cyclooxygenase have now been identified, the constitutive (COX-1) and the inducible (COX-2) forms, encoded by different genes (Merlie *et al.*, 1988; and Xie *et al.*, 1991). Differences in the NH_2-terminus or COOH-terminus of COX-1 and COX-2 appears to affect their subcellular localisation; COX-1 is found predominantly in the endoplasmic reticulum (ER) of prostanoid-forming cells, whereas COX-2 is in the nuclear membrane (Morita *et al.*, 1995). If the two isoenzymes occur in different subcellular compartments then it is possible that their respective metabolites would also be distributed in separate locations. For example, the arachidonic acid metabolites generated via COX-2 may preferentially distribute to the nuclear compartment of the cell and therefore be available to modulate transcription of target genes (DuBois *et al.*, 1996).

Cyclooxygenase-1 (COX-1)

COX-1 is a constituent enzyme of most tissues, regulating normal cell activity by synthesizing physiological amounts of prostaglandins. The prostaglandins so produced have a protective role in the organism by facilitating repair (Dewitt, 1991). In the main the concentration of COX-1 remains relatively stable, except when stimulated by hormones or growth factors, in which case 2- to 4-fold increases may occur.

COX-1 is a haemo- and glycoprotein with a molecular weight of 71kDa (Miyamoto *et al.*, 1976). The structure is that of a monotopic dimer, that is, the binding surface of the protein integrates into only one leaflet of the lipid bilayer of the ER (figure 1.5). The dimer is ellipsoidal in shape. The tertiary structure comprises three independent folding domains: an epidermal growth factor-like domain linked to the main body of the enzyme by a disulphide bridge, a second folding unit enabling insertion of the enzyme into the lipid bilayer and leading to the main body of the enzyme which forms the third

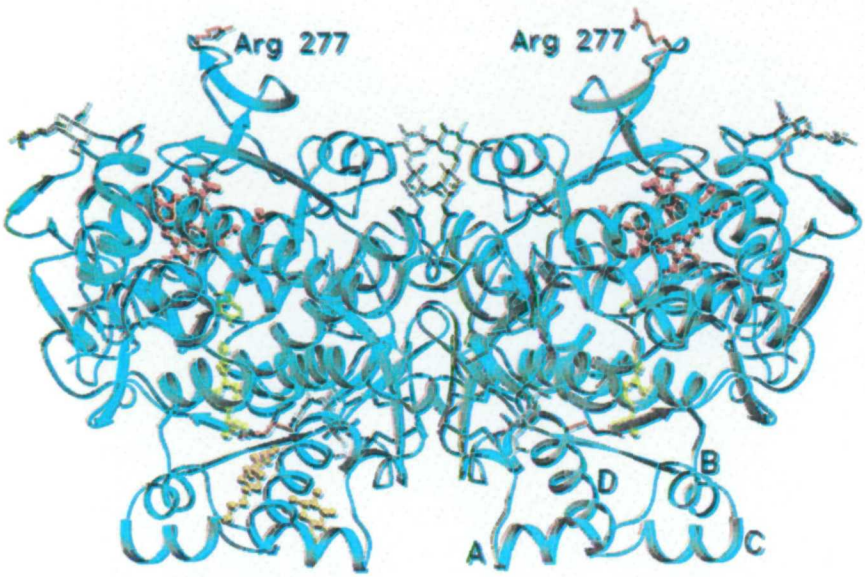

Figure 1.5 The structure of cyclooxygenase-1

This is a Molescript diagram of the COX-1 dimer derived from the 3.1Å refined structure. Haem groups are shown in red, Tyr 385 in green and flurbiprofen in yellow. Orange corresponds to a bound detergent molecule in the cyclooxygenase channel. Helices A, B, C and D of the membrane binding domain, and the site of trypsin cleavage (Arg 277), are also illustrated. Taken from Garavito (1996).

folding unit, a large, globular, catalytic domain. The catalytic domain is polar and therefore should be external to the membrane, with the bulk of the protein facing the lumen (Picot *et al.*, 1994).

It is within the catalytic domain that the cyclooxygenase and peroxidase active sites reside, adjacent but spatially distinct (Smith and Marnett, 1994). The cyclooxygenase active site consists of a long hydrophobic channel (8x25Å), extending from the outer surface of the membrane-binding domain to the centre of the protein. With the substrate arachidonic acid being hydrophobic, once released from membrane phospholipids, it will presumably remain associated with the bilayer, and so could feasibly gain direct access to the hydrophobic channel from the interior of the bilayer.

The residue tyrosine (Tyr) 385 is found at the apex of this channel, with serine (Ser) 530 lying just below it (Picot *et al.*, 1994). The active centre of cyclooxygenase is in the upper portion of the channel near Tyr 385. The only two polar residues within this otherwise hydrophobic channel are arginine (Arg) 120 and glutamine (Glu) 524; situated near to each other they probably interact by forming a salt bridge (figure 1.6).

Cyclooxygenase-2 (COX-2)

The gene encoding for a second cyclooxygenase enzyme, termed COX-2, was discovered serendipitously in 1991 (Xie *et al.*, 1991). Catalytically COX-2 is indistinguishable from COX-1 with respect to its function, substrate preference, enzyme kinetics, biochemistry and mechanism of prostaglandin biosynthesis (Loll and Garavito, 1994). The amino acid sequence of the cDNA of COX-2 has approximately 60% homology to that of COX-1 (O'Banion *et*

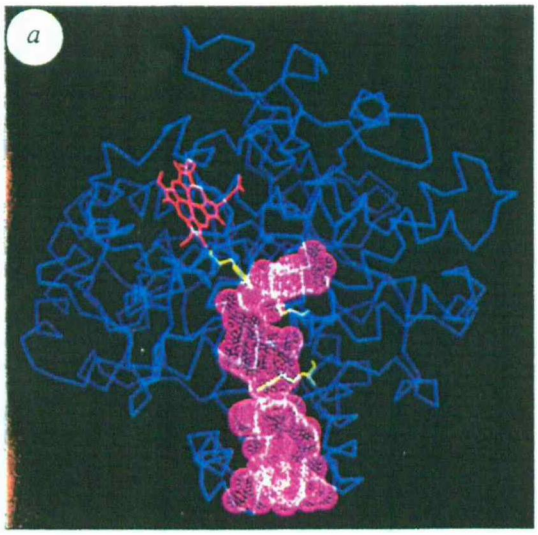

Figure 1.6 The cyclooxygenase channel and active site
This Van der Waals surface shows the hydrophobic cyclooxygenase channel extending within a monomer (blue) from the membrane-binding motif at the bottom to Tyr 385 (greenish yellow) at the top, just below the haem (red). The long axis of the channel passes between the large and small lobes of the catalytic domain. At the apex of the channel two small pockets extend to either side. Two other residues are also shown in greenish yellow: Ser 530 (at the top of the channel where it joins the pockets, just below Tyr 385) and Arg 120 (halfway up the channel). Taken from Picot *et al.* (1996).

al.,1992) and both enzymes have a molecular weight of 70kDa. The active site of COX-2 is larger than that of COX-1, and can accept a wider range of substrates. With the exception of the kidneys and the central nervous system, little or no COX-2 can be found in resting cells. However, COX-2 expression is increased markedly when fibroblasts, vascular smooth muscle or endothelial cells are exposed to growth factors, phorbol esters, cytokines, endotoxins or bacteria etc. The levels of COX-2 also increases in conditions of chronic inflammation, resulting in vastly elevated prostaglandin synthesis in many cells and tissues. Thus, COX-2 is induced in settings of inflammation by cytokines and inflammatory mediators.

1.5.3 Inhibition of Cyclooxygenase by NSAIDs

NSAIDs have been shown to inhibit the cyclooxygenase enzyme, thus preventing the synthesis of prostaglandins (Vane, 1971). NSAIDs bind at the top of the channel of COX, near to the putative catalytic residue Tyr 385 (Shimokawa *et al.*, 1990). The carboxylate group of the drug probably ligands to the guanidinium group of the Arg 120 residue in the channel (Picot *et al.*, 1994). NSAIDs such as flurbiprofen and indomethacin prevent substrate access by filling the entire channel from Tyr 385 downwards, a length of approximately 12-14Å. Aspirin, on the other hand, acetylates the residue Ser 530 in the channel. Although the acetyl group of aspirin would not fully occupy the channel, it does take up enough space to prevent the substrate gaining access to the upper portion of the channel and hence the active site. (figure 1.7).

This model would also explain the binding of the substrate arachidonic acid to the COX active site. The existence of Arg 120 in the channel would provide

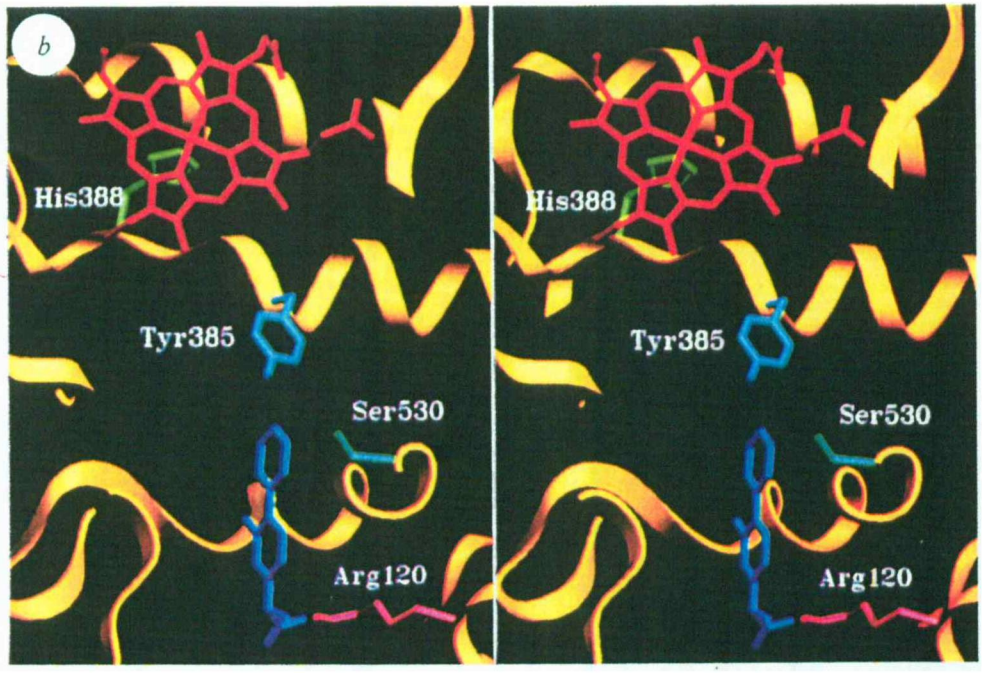

Figure 1.7 Interaction of cyclooxygenase with NSAIDs

The upper portion of the cyclooxygenase channel is shown in stereo as a ribbon representation with the NSAID flurbiprofen lodged into position. Tyr 385 (light blue) is at the apex of the channel, positioned near the haem. Ser 530 (violet) is also at the apex of the channel. Ser 530 is the site for aspirin acetylation, and although Ser 530 is not required for cyclooxygenase activity, aspirin acetylation may inhibit cyclooxygenase by blocking access of arachidonic acid to the active centre. Taken from Picot *et al.* (1996).

a suitable ligand for the carboxylate group of arachidonic acid to attach to whereas its C-13 would lie in a bent conformation in the vicinity of Tyr 385.

Mode of Inhibition

Enzyme inhibition can be either reversible or irreversible. An irreversible inhibitor dissociates very slowly from its target enzyme because it becomes tightly bound to the enzyme, either covalently or noncovalently. In reversible inhibition, on the other hand, there is rapid dissociation of the enzyme-substrate complex. Reversible inhibition can be further subdivided into competitive and noncompetitive. A competitive inhibitor normally resembles the substrate, and competes with the substrate for the active site of the enzyme. A noncompetitive inhibitor has a separate binding site to the active site; it acts by decreasing enzyme turnover rather than substrate binding.

In general, NSAIDs such as ibuprofen, inhibit cyclooxygenase by reversibly competing with its substrate arachidonic acid for the active site of the enzyme (Vane *et al.*, 1990). In the case of aspirin, however, the drug irreversibly inhibits cyclooxygenase by acetylation of Ser 530 on COX-1 or the Ser 516 on COX-2, thereby excluding access for arachidonic acid to Tyr 385 by steric hindrance (Roth *et al.*, 1975). Other NSAIDs such as indomethacin, flurbiprofen or meclofenamate cause an irreversible, time-dependent inhibition of cyclooxygenase (Loll and Garavito, 1994). That is, inhibition increases with time, probably by a non-covalent structural alteration of the enzyme-ligand complex creating a tighter complex.

It is possible that there are a number of subsites on COX for drug binding, some kinetically indistinguishable, since although the NSAIDs are a chemically diverse group of drugs, even compounds within the same

chemical family have some members which are reversible, competitive inhibitors whereas others are irreversible, time-dependent inhibitors of cyclooxygenase (Picot *et al.*, 1994). In addition, NSAIDs bear no close resemblance in structure to their substrate arachidonic acid (Smith and Marnett, 1994).

Whether cyclooxygenase is inhibited reversibly or irreversibly, the peroxidase is generally unaffected by NSAIDs (Kulmacz, 1989). That is, in the absence of cyclooxygenase activity peroxidase activity is still possible, though impairment of peroxidase activity of the enzyme would be accompanied by an impairment of cyclooxygenase activity.

Membrane Interaction

In order for the NSAID to reach its site of action, it needs to move from within the plasma compartment of the circulatory system, through tissue membrane structures, and eventually across the plasma membrane of the target cell, which usually contains relatively high amounts of cholesterol (Herbette *et al.*, 1996). The drug must then cross many intracellular barriers before being incorporated into the ER membrane network (COX-1), which has low levels of cholesterol, or further within the nuclear envelope (COX-2). NSAIDs must therefore have ampiphilic properties in order to be able to traverse several membrane barriers. Once within the membrane bilayer compartment, good membrane partitioning ability will allow the NSAID molecule to reach the intrabilayer active site of COX. Therefore, NSAIDs also need to be highly lipophilic in order to reside in the target membranes at sufficient concentrations to inhibit COX. Indeed, NSAIDs are generally planar, anionic molecules that partition into lipid environments, such as lipid bilayers of plasma membranes, and the more acidic the pH, such as at inflammatory sites, the greater the lipophilicity (Lombardino *et al.*, 1975).

COX-1 versus COX-2

The active site topographies of COX-1 and COX-2 have been shown to be subtly different in that during aspirin acetylation, there is almost a complete block of COX-1 activity, but in the slightly larger channel of COX-2 the acetyl-serine side-chain can rotate, thus allowing limited access of arachidonic acid to the active site. Therefore, even when acetylated by aspirin, COX-2 has been shown to oxygenate arachidonic acid, producing 15-HPETE, though not PGG_2, which is then converted to 15-HETE by the COX peroxidase (Lecomte *et al.*, 1994).

Evidence suggests that COX-1 has a 'housekeeping' function, generating prostaglandins necessary for normal cellular processes, whereas COX-2 is responsible for producing prostaglandins involved in inflammation and growth regulation. Therefore, the unwanted side-effects of NSAIDs such as gastrointestinal lesions, is thought to be due the inhibition of COX-1, while their main antiinflammatory effects are due to inhibition of COX-2 (figure 1.8; and Herschman, 1994). Studies are currently being undertaken to identify NSAIDs which reduce inflammation but spare the associated side effects (Yang, 1996). However, it is obvious that further work needs to carried out to understand the precise role of COX in gastric pathophysiology since mouse deficient in the COX-1 gene gave an apparent paradoxic decrease in their susceptibility to NSAID-induced gastric damage (Langenbach *et al.*, 1995).

1.5.4 The Role of Prostaglandins

Inflammation

Inflammation can be thought of as essentially a protective response to noxious stimuli such as physical injury (mechanical trauma, irradiation, cold

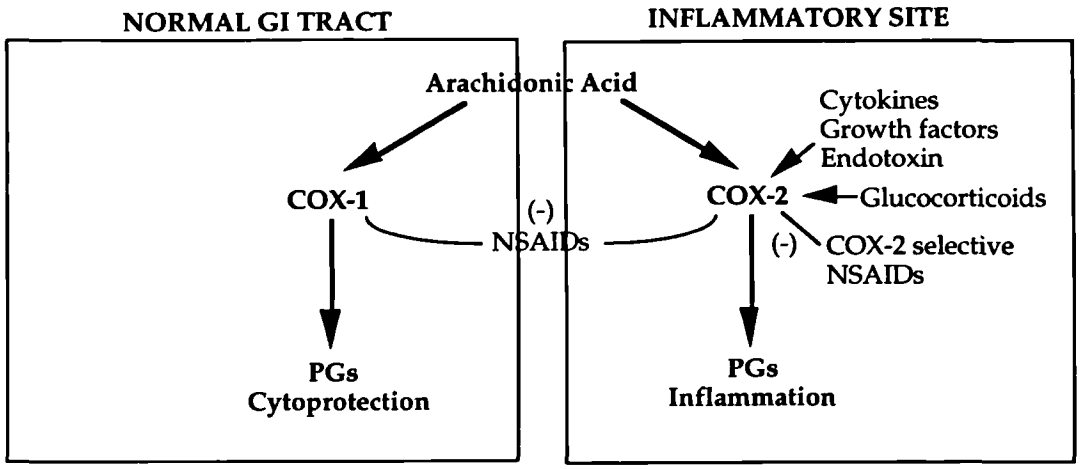

Figure 1.8 Proposed role of COX-1 and COX-2 in normal and inflamed tissues

Under normal physiological conditions, the gastrointestinal (GI) tract synthesizes cytoprotective prostaglandins (PGs) via cyclooxygenase-1 (COX-1). Inhibition of COX-1 by NSAIDs results in gastric lesions. During inflammation cytokines stimulate COX-2 which produces large amounts of pro-inflammatory prostaglandins, pain and oedema. COX-2 selective NSAIDs are anti-inflammatory without GI side-effects seen with traditional NSAIDs. Adapted from Masferrer *et al.* (1996).

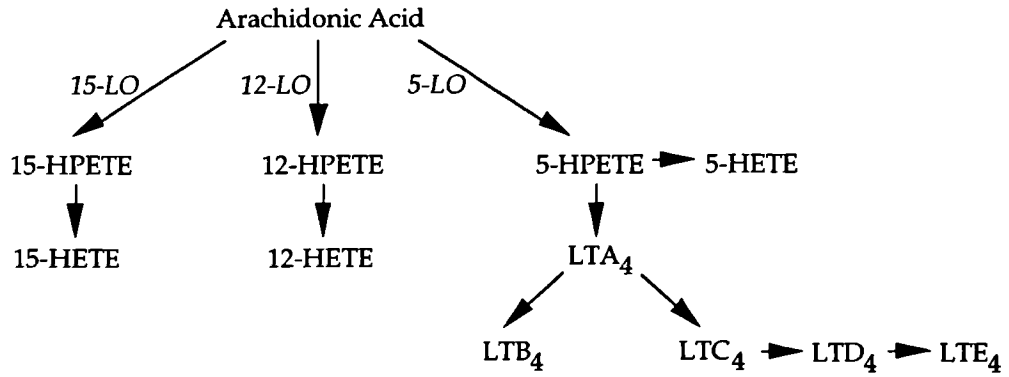

Figure 1.9 Lipoxygenase biosynthesis

In addition to cyclooxygenase, arachidonic acid can also be metabolised by lipoxygenase to form leukotrienes. See main text for abbreviations.

or heat), chemical injury (acids, alkalis, phenols or other inflammatory irritants) and injury due to a living organism (viruses, bacteria, fungi, parasites). The characteristic signs of inflammation are erythema, oedema (swelling), tenderness (hyperalgesia) and pain.

The defense mechanisms involved in the inflammatory response include firstly the recognition of the foreign material, followed by the generation of mediators (such as leukotrienes, platelet-activating factor and prostaglandins) and cytokines (such as tumour necrosis factor, interleukin-1 and interleukin-8) from immunocytes (mast cells, macrophages, neutrophils etc), accumulation of phagocytes and finally activation of phagocytes at the site of inflammation (Wallace and Tigley, 1995). For example, when an organism is re-exposed to an antigen to which it has been previously sensitised, mast cells are activated, releasing the mediators histamine and leukotrienes. In the case of leukotriene B_4, this would be responsible for recruiting neutrophils from the vasculature into the tissues, an important defensive response. Moreover, both mast cells and macrophages can act as cytotoxic cells, using nitric oxide and/or tumour necrosis factor to kill other cells.

Under normal conditions macrophages, neutrophils, fibroblasts and endothelial cells show very little cyclooxygenase activity and thus no prostaglandin production. During inflammation, however, several phospholipases are activated in the inflamed tissue and infiltrating cells, liberating large amounts of free arachidonic acid. Likewise, cyclooxygenase activity is stimulated. It is prostaglandin E_2 and prostacyclin which are the predominant cyclooxygenase products found in acute inflammatory exudates and have potent pro-inflammatory actions (Whittle, 1992). These prostaglandins play a role in modulating, or down-regulating, the activity of many immunocytes, including macrophages and mast cells, and hence the

inflammatory response. For example, PGE_2 has been shown to be a potent suppresser of TNF release from macrophages (Kunkel *et al.*, 1986a) and also to modulate the expression of the TNF gene in macrophages (Kunkel *et al.*, 1988). Prostaglandins can also regulate the release of other cytokines, such as interleukin-1, from macrophages (Kunkel *et al.*, 1986b). Release of the mediators TNF, PAF and histamine from mast cells can be inhibited by E-type prostaglandins (Hogaboam *et al.*, 1993).

During inflammation, neutrophils are recruited to the site(s) of injury by the chemical mediators (chemotaxins) released from immunocytes. Prostaglandins can suppress the release of LTB_4 (Ham *et al.*, 1983) and IL-8 (Wertheim *et al.*, 1993) from neutrophils, and the adherence of neutrophils to the vascular endothelium (Granger and Kubes, 1994).

In the gastrointestinal tract eicosanoids together with other substances such as cytokines, reactive oxygen metabolites, platelet activating factor and the bacterial chemotactic peptide fMLP modulate host responses (Powell, 1991). In inflammatory bowel disease the levels of eicosanoids PGE_2, LTB_4, and perhaps LTC_4, 12-HETE, 15-HETE and TXA_2 are increased in inflamed tissues compared with normal mucosa, and return to normal during remission (Ebehart and DuBois, 1995).

Involvement of COX-2 in inflammation

It is the activity of COX-2, as opposed to COX-1, which increases in response to an inflammatory stimulus. The expression of the COX-2 gene is responsible for a transient production of proinflammatory prostaglandins.

Many studies have implicated COX-2 in inflammation. One of the first and more important observations that supported the existence of an inducible

cyclooxygenase enzyme was found when its expression was increased following incubation with the pro-inflammatory cytokine IL-1β (O'Banion *et al.*, 1992) and TNFα (O'Banion *et al.*, 1994). Pharmacological intervention with the antiinflammatory glucocorticoid dexamethasone could block the expression of this inducible cyclooxygenase without effect on basal cyclooxygenase activity, whereas the NSAID indomethacin inhibited both basal and stimulated cyclooxygenase activity (Masferrer *et al.*, 1994). In response to inflammatory stimuli, COX-2 expression is readily induced *in vitro* and *in vivo* in cells such as macrophages, monocytes (Fu *et al.*, 1990), synovial cells (Crofford *et al.*, 1994), and endothelial cells (Habib *et al.*, 1993). Furthermore, in animal models of inflammation, cyclooxygenase activity and expression have been shown to be markedly increased. For example, Vane and associates (1994) demonstrated a significant increase in COX-2 mRNA and protein in a murine air pouch model of granulomatus inflammation, while the COX-1 protein levels remained unchanged. In a model of acute inflammation, such as carrageenan injection into the footpad, a marked accumulation of COX-2 mRNA was reported plus a selective inhibitor of COX-2 (SC-58125) inhibited oedema at the inflammatory site and was also shown to be analgesic. Again COX-1 expression was unaltered during the inflammatory process (Seibert *et al.*, 1994). Similarly in adjuvant-induced arthritis an induction of COX-2 mRNA and protein correlated with the rise in prostaglandin levels in the inflamed joint and the incidence of oedema. By selectively inhibiting COX-2 and the production of these prostaglandins, the oedema was attenuated in these chronically inflamed animals (Crofford *et al.*, 1994).

Vascular events in inflammation

Acute inflammation is characterized by vasodilatation, increased vascular permeability, accumulation of leukocytes and pain. Certain prostaglandins are

potent vasodilators. Prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2) produce a long-lasting (up to 10 hours) erythema, seen in acute inflammation (Solomon *et al.*, 1968). During inflammation, the prostaglandins are generated locally and cause vasodilatation by acting directly on vascular smooth muscle by relaxing vascular tone (Williams, 1985). The prolonged vascular effects of stable prostaglandins and their ability to counteract the vasoconstriction caused by agents such as noradrenaline and angiotensin, are properties not shared by the other inflammatory mediators (Higgs *et al.*, 1980).

Prostaglandins themselves do not increase vascular permeability, but PGE_1, PGE_2 and PGI_1 are very potent at enhancing the oedema induced by other inflammatory mediators such as bradykinin and histamine (Moncada *et al.*, 1973). This is possibly due to the arteriolar dilatation caused by prostaglandins, increasing hydrostatic pressure within venules (Johnston *et al.*, 1976).

Pain and Hyperalgesia

The pain that accompanies inflammation and tissue injury is caused by local stimulation of pain fibres and enhanced pain sensitivity (hyperalgesia) due to the increased excitability of the central neurones in the spinal cord (Konttinen *et al.*, 1994). Bradykinin and cytokines such as IL-1, TNF and IL-8 are thought to be involved in eliciting the peripheral pain of inflammation. Prostaglandins (PGE_2 and PGI_2) can sensitize afferent pain receptors to normally painless mechanical or chemical stimulation, by lowering the threshold of polymodal noiciceptors (peripheral sensory nerves) of C fibres. However, although NSAIDs are particularly effective in the relief of the pain of inflammation, this again may not be only by reducing prostaglandin production, but the drugs may also have an antinociceptive effect at peripheral or central neurones (Gebhart and McCormack, 1994).

Fever

Fever results from various disease states including inflammation, malignancy, graft rejection, tissue damage (Dascombe, 1985). During fever there is an increased production of cytokines IL-1 and IL-6, interferons α and β, and $TNF\alpha$. The cytokines cause an increase in the synthesis of PGE_2, via increases in cyclic AMP, and PGE_2 then stimulates the hypothalamus to elevate body temperature by promoting an increase in heat production and a decrease in heat loss. NSAIDs bring down a raised body temperature by inhibiting PGE_2.

Dysmenorrhoea

The pathogenesis of dysmenorrhoea is thought to be due to increased production and release of endometrial prostaglandins during menstruation, which results in increased and abnormal uterine activity (Dawood, 1981). During the ovulatory cycle, the corpus luteum undergoes regression and the resulting decrease in progesterone levels gives rise to lysosomal labilization, thereby releasing the lysosomal enzyme phospholipase A_2. Phospholipase A_2 hydrolyses cell membrane phospholipids in the endometrium producing arachidonic acid. The availability of arachidonic acid together with the cellular trauma that occurs in the endometrium during the onset of menstruation, drives the arachidonic acid cascade towards prostaglandin production. In dysmenorrhoea, the increased levels of prostaglandins $PGF_{2\alpha}$ and PGE_2 in the menstrual fluid, stimulates the myometrium causing increased and abnormal myometrial contractions. As a result, uterine blood flow decreases and uterine ischaemia develops.

Thus, prostaglandins are responsible for the labour-like pain and contractions as well as the diarrhoea, nausea and vomiting seen in dysmenorrhoea. The production and release of prostaglandins occurs mainly during the first 48

hours of menstrual flow, explaining why in dysmenorrhoea the intense pain is normally felt in the first or second day of menstruation. NSAIDs relieve dysmenorrhoea by suppressing prostaglandin production in menstrual fluid. The failure of aspirin to relieve the symptoms of dysmenorrhoea may be due to its rapid hydrolysis, consequently insufficient drug reaches the cells of the uterus to inhibit cyclooxygenase (Mustard, 1982).

Patent Ductus Arteriosus

Prostaglandins are important in maintaining the patency of the ductus arteriosus (Coceani and Olley, 1973). During parturition, the blood oxygen increases and prostaglandin production drops, one of the factors in the closure of the ductus arteriosus. Administration of NSAIDs at doses that inhibit prostaglandin formation can also cause closure of the patent ductus, although the drugs may sometimes cause premature closure when given late in pregnancy (Sharpe *et al.*, 1974).

Labour

The use of NSAIDs such as indomethacin to prevent preterm labour is based on their ability to suppress the formation of prostaglandins. During labour the prostaglandins E_1, E_2, $F_{1\alpha}$ and $F_{2\alpha}$ appear in large quantities in amniotic fluid and maternal blood, the most abundant being the F prostaglandins (Karim and Devlin, 1967). These prostaglandins probably originate from the uterine endometrium which, during labour, synthesizes them at a higher than normal rate. The prostaglandins initiate labour by causing uterine contraction with the uterus being more sensitive to prostaglandins in late pregnancy.

Cardiovascular Diseases

Thromboxane A_2 causes platelet aggregation forming a white thrombus, which obstructs coronary or cerebral arteries, eventually leading to a heart attack or stroke (Collier, 1984). The ability of aspirin to protect against coronary infarction and stroke is by way of its virtue of preventing blood platelets generating thromboxane A_2 from arachidonic acid without preventing production of PGI_2 from endothelial cells.

Pregnancy

Excess production of TXA_2 and PGI_2 has been implicated in the genesis of preeclampsia and pregnancy-induced hypertension (Lubbe, 1987). Aspirin has been shown to prevent both these conditions by inhibiting prostaglandin synthesis.

1.5.5 Prostaglandin-Independent Mechanism of Action of NSAIDs

Although generally it is accepted that the mechanism of action NSAIDs is due to their ability to inhibit cyclooxygenase, this may not account for all the effects of NSAIDs. For example, sodium salicylate is equipotent with aspirin in its antirheumatic effects, but does not inhibit cyclooxygenase (Ferreira, 1977). Although relatively low doses of NSAIDs are required to inhibit prostaglandin biosynthesis, higher doses are required for an antiinflammatory effect (Higgs *et al.*, 1976b), suggesting that the antiinflammatory activity exhibited by NSAIDs at higher concentrations is independent of their ability to inhibit cyclooxygenase (Abramson *et al.*, 1985).

Inhibition of Lipoxygenase

In addition to the cyclooxygenase pathway, arachidonic acid can also be metabolised by lipoxygenase (LO) to form leukotrienes which have an

important role in inflammatory processes (figure 1.9; and Bray, 1986). NSAIDs can inhibit lipoxygenase, thereby stimulating the reincorporation of arachidonic acid into triglycerides and decreasing the production of leukotrienes from leukocytes and synovial cells (Ku *et al.*, 1985). All NSAIDs appear to inhibit 15- and 12-lipoxygenase (Siegel *et al.*, 1980).

The end product of the lipoxygenase pathway in human platelets is 12*L*-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE), with the unstable intermediate being 12*L*-hydroperoxy-5,8,10,14-eicosatetraenoic acid (HPETE). Aspirin and indomethacin have been shown to inhibit the conversion of HPETE to HETE in human platelets via a putative HPETE peroxidase activity (Siegel *et al.*, 1979). Thus, the pharmacological actions of aspirin, indomethacin and other NSAIDs may be related to their property of causing an accumulation of HPETE at the expense of enzymatic HETE production. For example, HPETE analogues, by inhibiting prostacyclin synthetase inhibit the formation of prostacyclin which causes hyperalgesia; this is a possible explanation for the analgesic effects of NSAIDs (Moncada *et al.*, 1976). The antiinflammatory activity of the drugs, on the other hand, may partly be due to the fact that HETE is chemotactic, therefore inhibition of its production by NSAIDs will prevent the recruitment of phagocytic cells (Vinegar *et al.*, 1973).

Inhibition of Neutrophil Function

At high (antirheumatic) concentrations, NSAIDs inhibit neutrophil activation (Kaplan *et al.*, 1984). Abramson *et al.* (1985) showed that aspirin, ibuprofen, piroxicam and indomethacin all inhibited neutrophil aggregation, lysosomal enzyme release, and superoxide generation, albeit to varying extents. They suggested this effect was not due to inhibition of prostaglandin synthesis since addition of exogenous prostaglandin to NSAID-treated neutrophils did not restore the cell responses. Moreover, sodium salicylate,

which does not inhibit cyclooxygenase, was as effective an inhibitor of neutrophil aggregation as was aspirin. Therefore, it was postulated that inhibition of neutrophil activation by NSAIDs occurred independently to that of prostaglandin synthesis, probably by inhibiting calcium translocations, the early signals in the activation of neutrophils.

In addition, NSAIDs have been shown to inhibit discrete neutrophil responses such as aggregation, superoxide generation, and lysosomal enzyme release induced by the chemotactic peptides fMLP, concanavalin-A, and phorbol myristate acetate, which participate in the inflammation and tissue destruction associated with rheumatoid arthritis (Abramson *et al.*, 1983; and Forrest and Brooks, 1988). It has been suggested that these effects are independent of the prostaglandin inhibition caused by the NSAIDs.

Interference With Leukocyte Migration

There is a variation in the effect of NSAIDs on the migration of inflammatory cells. For example, ibuprofen and benoxaprofen inhibit monocyte chemotaxis *in vivo* whereas indomethacin, naproxen and salicylate do not. Similarly, several NSAIDs have been shown to significantly inhibit polymorphonuclear leukocyte chemotaxis, but indomethacin has not (Dawson, 1980). If this was a prostaglandin-mediated response then all NSAIDs should inhibit migration as they all inhibit cyclooxygenase (Goodwin, 1984). Several studies have substantiated the lack of correlation between the dose-response curve for this effect and inhibition of prostaglandin production by NSAIDs (Ford-Hutchinson *et al.*, 1979; and Adams *et al.*, 1977).

1.6 Side-effects of NSAIDs

NSAIDs appear to induce toxic reactions in almost all of the major organ systems in man, albeit to varying severity and frequency (Rainsford, 1984). The scale of the problem is immense, for example, over the past 15 years or so, one quarter of all adverse drug reactions reported to the Committee on Safety of Medicines in the United Kingdom, have been due to NSAIDs (Mann, 1987). This equates to approximately 30,000 major complications a year. For arthritic patients, in particular, who have to take NSAIDs in large quantities and for a long period of time, the frequent occurrence of side-effects often warrants interruption or discontinuation of the use of these drugs (Rainsford, 1989a). Even more worrying is the relatively high incidence of deaths associated with the use of NSAIDs (expressed as annual deaths per million prescriptions): oxyphenbutazone (14.3), aspirin (11.5), phenylbutazone (7.1), and indomethacin (4.4) (Girdwood, 1974).

Generally, the adverse effects are dose-related, though some are idiosyncratic, and largely (but not solely) attributable to inhibition of prostaglandin synthesis (Rainsford, 1988). Some of the most common unwanted effects of NSAIDs are summarised in table 1.10.

The most frequently seen adverse effects of NSAIDs, especially patients taking the drugs long-term and in the elderly, is gastrointestinal (GI) upset. The proposed mechanism of GI damage will be discussed in detail in the next section.

Another major target for NSAID toxicity is the kidney. PGE_2 and PGI_2 are vasodilatory. In the kidneys, these prostaglandins are produced in response to

Table 1.10 Common adverse effects of NSAIDs

Adverse Effects
Gastrointestinal
Ulceration
Perforation
Haemorrhage
Renal
Acute renal failure
Hypertension
Cardiac failure
Hypersensitivity
Rashes
Bronchospasm
Haematological
Thrombocytopenia
Neutropenia
Red cell aplasia
Haemolytic anaemia
Neurological
Headache
Tinnitus
Dizziness
Blurred vision
Personality changes (irritability, hyperactivity, nervousness, drowsiness and depression)

Adapted from Thompson and Dunne (1995), and Mortensen and Rennebohm (1989).

the vasoconstrictor hormones noradrenaline, angiotensin II and vasopressin, in order to antagonise the renal vasoconstriction and renal ischaemia caused by the hormones, by a negative feedback loop. The prostaglandins thereby act by maintaining renal blood flow and glomerular filtration rate (GFR) which, in healthy individuals, is not very important as there is a considerable reserve capacity for GFR. A decrease in prostaglandin production by NSAIDs in conditions where there is already reduced GFR e.g. shock, congestive heart failure, hepatic cirrhosis with ascites, chronic renal disease, and in hypovoleamic individuals, will cause significant changes in renal function (i.e. further reduction in GFR), which may then lead to acute renal failure (Clive and Stoff, 1984). By inhibiting the production of renal prostaglandins, NSAIDs decrease renal perfusion in these patients, thus acute renal failure may result. NSAIDs also promote salt and water retention by inhibiting prostaglandin-induced chloride reabsorption and the action of antidiuretic hormone. Therefore, in patients treated with NSAIDs, oedema may occur and the drugs may also reduce the effectiveness of antihypertensive regimens (Oates *et al.*, 1988).

NSAIDs have also been shown to cause hypersensitivity, due to intolerance, in 20-25% of middle-aged patients with asthma, nasal polyps or chronic urticaria, with symptoms resembling those of anaphylaxis (Ng, 1992). The underlying mechanism for this hypersensitivity reaction to NSAIDs is unknown, although one theory is that by inhibiting cyclooxygenase, arachidonic acid metabolism is diverted toward the formation of leukotrienes by the lipoxygenase pathway. This hypothesis is based on the fact that leukotrienes C_4 and D_4 may precipitate bronchial spasm and that inhibition of 5-lipoxygenase by the drug zileuton, may prevent the signs and symptoms of aspirin intolerance (Israel *et al.*, 1993). Alternatively, NSAID-induced

hypersensitivity may be caused by a perturbation of cellular immune reactions (O'Brien and Bagby, 1985).

Haematological disturbances are also often seen with NSAIDs, in particular an increase in bleeding time (Insel, 1996). This is due to their ability to prevent the formation by platelets of thromboxane A_2 (TXA_2), a potent platelet aggregator. Aspirin is a particularly effective inhibitor of platelet function due to its irreversible inhibition of cyclooxygenase; new platelet needs to be produced in order to restore enzyme activity. This effect of aspirin is exploited in the prophylaxis treatment of cardiovascular diseases.

1.6.1 Mechanism of Gastrointestinal Damage

The most common side-effects of NSAIDs is seen in the gastrointestinal tract, and these range from mild dyspepsia, heartburn, constipation and diarrhoea (symptomatic reactions) to the potentially life-threatening effects of ulceration and haemorrhage (Rainsford, 1987). Ironically, it was the discovery that aspirin induced GI damage which was responsible for the proliferation of NSAIDs, in an effort to find newer compounds with good anti-inflammatory and analgesic properties but with reduced GI toxicity (Borda, 1992). Both in animal models (Cucala *et al.*,1987) and healthy human volunteers (Lanza, 1984), there is a strong correlation between the antiinflammatory potency of NSAIDs and their gastric ulcerogenicity. The effects of NSAIDs on the GI tract are both local and systemic.

Local Effects of NSAIDs

According to data from rats, the local effect of the NSAIDs on the gastric mucosa occurs within 15 to 60 minutes of oral administration and involves extensive shedding of superficial mucous cells in the glandular mucosa,

followed by the development of endoscopically visible erosions in the upper glandular mucosal region and subsequent haemorrhage in the eroded areas (Rainsford, 1989b). (Maximal development of lesions may take longer with those NSAIDs which have a slower absorption into the gastric mucosa (Rainsford *et al.*, 1984)). The local effect occurs by the phenomenon of "ion-trapping" (see figure 1.2). At the low pH in the stomach, the NSAID (a weak acid) is almost totally unionised and, being also highly lipophilic, freely partitions into the cells of the gastric epithelium (Schoen and Vender, 1989). Once within the cells, which have a neutral pH, the drug reionises and becomes trapped at high concentrations. Direct cellular injury is presumably caused by the toxicity of high intracellular levels of the NSAID. These toxic effects lead to the cell membrane permeability becoming altered, enabling back diffusion of acid thereby damaging the deeper layers of the gastric mucosa. This phenomenon may explain why aspirin is much more gastrotoxic than other NSAIDs. That is, aspirin has a higher solubility in the acidic pH conditions of the stomach than most other NSAIDs, and so more readily enters the cells of the stomach wall (Beck *et al.*, 1990). Highly buffered aspirin, enteric-coated aspirin or naproxen have been formulated to somewhat lessen their topical irritation.

Systemic Effects of NSAIDs - Prostaglandin Inhibition

The fact that not just orally but parenterally administered NSAIDs also cause gastric damage, suggests that factors other than the local effects of cell exfoliation and back diffusion of acid from the stomach lumen into the mucosa of the stomach wall, also apply. The systemic effect of NSAIDs is thought to be via the inhibition of cyclooxygenase both COX-1 and COX-2, leading to both beneficial (anti-inflammatory and antipyretic) and toxic (gastrointestinal injury) effects. Nevertheless, it is the inhibition of the

synthesis of gastroprotective prostaglandins, now hypothesized to be COX-1 mediated, which plays a role in NSAID-induced gastrointestinal damage.

Mucus and bicarbonate secretion

Prostaglandins are important in mucosal defense by stimulating mucus (McQueen *et al.*, 1983) and bicarbonate (Kauffman *et al.*, 1980) secretion. Mucus slows down the diffusion of luminal gastric acid and secretion of bicarbonate neutralizes it. The mucus, a mixture of mucin, other proteins and lipids, is secreted by gastric and duodenal epithelial cells forming a polymeric gel into which bicarbonate is secreted by these cells, thereby providing a protective pH gradient that maintains a neutral pH at the epithelial cell surface (Quigley and Turnberg, 1987). Mucin is a viscous glycoprotein whose physicochemical properties provide a relatively acid-resistant barrier. Gastric acid is secreted in a pulsatile manner through the mucus gel forming temporary channels within the gel, and these rapidly close to prevent luminal acid back-diffusion. This mechanism maintains a neutral pH at the mucosa despite the low luminal pH.

In addition, gastric mucus also acts as a physical barrier by lubricating the mucosal surface to allow free passage of solid particles whilst at the same time protecting the surface from mechanical damage by such material (Whittle, 1984). It has also been suggested that the mucus layer may even thicken in response to injury, enabling re-epithelialization of the mucosa (Morris *et al.*, 1984).

In the gastric mucosa, a mucoid cap, consisting of mucus, lipids, fibrin and cellular debris, forms over an area of superficial damage, providing an alkaline microenvironment required for mucosal restitution (Wallace and Whittle, 1986). The pH gradient across this cap is produced by bicarbonate

secretion and plasma flux. Together with mucus and bicarbonate secretion, the repair of superficial injury is also enhanced by prostaglandins, which maintain endothelial integrity by stimulating cell proliferation and migration along the basement membrane (Tarnawski *et al.*, 1985).

Enhance mucosal blood flow

E and I prostaglandins enhance mucosal blood flow, which is beneficial in maintaining the cellular and functional integrity of the gastric tissue (Main and Whittle, 1973). The blood supplies essential nutrients, oxygen, and gastrointestinal hormones to support the upper structure, function and turnover of the gastric mucosa. The blood also prevents intramucosal accumulation of hydrogen ions, metabolic waste, and other potential toxins and sustains bicarbonate secretion, thereby protecting the mucosa by maintaining a neutral pH in the regional mucosa. In fact, irritants stimulate prostaglandin-induced vasodilatation but when this is prevented erosions develop (Whittle, 1977).

Other mechanisms

At extremely low concentrations, prostaglandins can inhibit pro-inflammatory mediator release, such as histamine, by acting directly on mucosal mast cells (Hogaboam *et al.*, 1993). As a result, there is a reduction in the acidity, volume and pepsin content of gastric secretions and also the defensive hyperaemic responses to luminal irritants. Prostaglandins of the E and I type inhibit the production of reactive oxygen species production and enzyme release by neutrophils, found to be important in gastric damage (Kainoh *et al.*, 1990). Prostaglandins also appear to increase the production of phospholipid molecules at the surface of the gastric epithelium, which may repel fluid and provide some degree of protection (Lichtenberger *et al.*, 1985).

Finally, prostaglandins can stabilize lysosomal membranes (Ferguson *et al.*, 1973).

Consequence of PG inhibiton by NSAIDs

By inhibiting of the synthesis of these prostaglandins, NSAIDs interfere with gastric mucosal defense. Indeed, pretreatment with NSAIDs certainly predisposes the mucosa to injury by topical irritants (Whittle, 1983). Many lines of evidence show that NSAIDs are responsible for forming a mucus gel poorly suited as a barrier to acid diffusion. Aspirin and other NSAIDs can reduce mucus secretion (Garner and Allen, 1987), its viscosity (Sarosiek *et al.*, 1986) and the biosynthesis of mucus glycoproteins (Rainsford, 1978). Aspirin and indomethacin have also been reported to suppress basal bicarbonate secretion from gastric and duodenal mucosa, which can be reversed on administration of exogenous PGE_2 (Selling *et al.*, 1987). Inhibition of mucus and bicarbonate secretion by aspirin causes a substantial reduction in the hydrophobicity of the mucosal surface (Goddard *et al.*, 1990). As it is believed that it is the water-repellent properties of the luminal surface which protects the mucosa from attack by the luminal acid, such changes by aspirin and other NSAIDs could, therefore, augment back-diffusion of acid and contribute to the local irritant effects of these drugs (Whittle, 1992). Additionally, NSAIDs may impair mucosal defense by decreasing the feedback inhibition of gastric acid secretion dependent on prostaglandins, and thereby increasing acid secretion (Taylor *et al.*, 1994). NSAIDs also prevent epithelial repair by dissipating the pH gradient across the mucoid cap, thereby reducing its effectiveness (Wallace and McKnight, 1990).

Mucosal injury is associated with focal areas of decreased blood flow, and experimentally a reduction in mucosal blood flow has been shown as an early event in NSAID-induced mucosal injury (Kitahora and Guth, 1987). NSAIDs

induce microvascular ischaemia, partly by causing neutrophil adherence to the vascular endothelium of the gastric microcirculation, and this can be blocked by exogenous prostaglandins (Wallace *et al.*, 1990).

Prostaglandin-Independent Mechanism of NSAID-induced Gastrointestinal Injury

Lack of correlation between PG inhibition and gastrointestinal damage

It is uncertain that the gastrointestinal injury caused by NSAIDs is due solely to inhibition of prostaglandin synthesis as several recent studies have shown no correlation between reduction in mucosal prostaglandin biosynthesis by NSAIDs and degree of mucosal injury. For example, aspirin given parenterally (100 mg/kg) or orally (25 mg/kg) to rats did not produce acute mucosal lesions despite inhibiting prostaglandin generation by up to 95% (Ligumsky *et al.*, 1983). Only by increasing the dose of aspirin 4-fold and administering it orally, did the drug produce mucosal injury. It was suggested that inhibition of cyclooxygenase activity may be a prerequisite for lesion formation, but alone is not the cause of aspirin-induced damage. The mechanism of injury may involve topical irritation by high dose of aspirin at the cellular level. Evidence of a direct mucosal irritant effect of aspirin can be seen by light or electron microscopy of human gastric mucosa, which shows signs of ultrastructural damage within 5-10 minutes of oral ingestion of the drug, too short a time course for prostaglandin inhibition (Baskin *et al.*, 1976). Furthermore, omeprazole can abolish aspirin-induced gastric mucosal lesions, presumably because omeprazole raises the intragastric pH to 5.6-6.9 (Scheiman *et al.*, 1994). Therefore, aspirin which has a pK_a of 3.5 is totally ionised (less than 1% unionised) and unable to diffuse passively into the gastric cells and evoke damage by "ion-trapping".

In another study, aspirin was reported to cause structural changes to the basement membrane of capillary and postcapillary venules in the rat stomach before damaging the surface epithelium (Rowe *et al.*, 1987). It was postulated that the vascular injury brought about by aspirin is possibly a result of it being hydrolysed *in vivo* to salicylic acid and secondary to the inhibition of prostacyclin in the vessel wall or thromboxane in the platelets. Salicylic acid causes direct toxic effects on the epithelial and endothelial cells producing haemorrhage into the tissue or vascular thrombosis by disruption of the endothelial cells, and these effects cannot be prevented by the administration of exogenous prostaglandins. As already mentioned aspirin, as with other NSAIDs, are thought to damage the gastric mucosa by two mechanisms, inhibition of prostaglandin synthesis and direct irritant action. It is possible, therefore, that the topical irritation caused by aspirin is partly due to salicylic acid formed on hydrolysis.

A lack of correlation was also found with oral ingestion of indomethacin (50 mg for 4 days) by healthy human subjects, which caused a significant reduction in PGE_2 and $PGF_{2\alpha}$ in the fundic and antral regions of the stomach without any apparent mucosal injury (Redfern *et al.*, 1987). Again, higher doses of the drug (a single 100mg dose) did produce mucosal lesions, but did not further reduce the mucosal concentrations of the prostaglandins. Similar observations were reported by Ligumsky and coworkers (1990), who showed that rectal administration of only indomethacin (10, 20 and 30 mg/kg) and diclofenac (10, 20 and 50 mg/kg) induced significant gastric damage in rats whereas ibuprofen did so only at its highest dose (100 mg/kg). Sulindac (10, 20 and 30 mg/kg) and aspirin (50, 100 and 200 mg/kg) failed to cause any lesions at all, despite the drugs inhibiting PGE_2 to the same extent.

Levine *et al.* (1988) showed that the NSAID carprofen produced less damage to the upper gastrointestinal tract despite having comparable antiinflammatory, analgesic and antipyretic effects to indomethacin in man, and being as effective at inhibiting prostaglandin synthesis.

NSAID-induced small intestinal damage

Most NSAIDs are known to damage the stomach mucosa. However, an increasing number of reports suggest that these drugs also cause lesions of the small intestine. In fact up to 70% of patients receiving NSAIDs for 6 months or more have evidence of small-intestinal inflammation as a consequence of increased mucosal permeability (Bjarnason *et al.*, 1987a). Blood loss is commonly seen, possibly leading to iron deficiency anaemia, as is protein loss, which may cause hypoalbuminaemia in some patients. The drugs also cause ileal dysfunction, reflected as a malabsorption of bile acids. More rarely, strictures of the small bowel occur (Bjarnason *et al.*, 1988). The inflammation and the biochemical abnormalities can persist for up to 16 months after discontinuation of the NSAIDs (Bjarnason and Macpherson, 1989).

High local concentrations of the NSAID are required to induce small-intestinal lesions. It appears that the damage is partially related to enterohepatic circulation of the NSAID since drugs not undergoing this process have been shown not to develop lesions (Reuter *et al.*, 1997). Enterohepatic circulation is created when the drug is conjugated in the liver, eliminated via the bile, then the acylglucuronide conjugates hydrolysed in the small intestine by the action of enteric bacterial β-glucuronidases. The parent compounds are then reabsorbed lower in the alimentary canal where these free acids will irritate the mucosal surface. By undergoing enterohepatic circulation there is long-term exposure of the small intestinal mucosa to the noxious effect of the NSAID (Duggan *et al.*, 1975; and Brune *et al.*, 1985).

However, this may also result in prolonged prostaglandin inhibition and damage (Brune *et al.*, 1988).

In addition, the near neutral pH of the intestine together with increased intestinal permeability, favours entry of bacterial or foreign enteric antigens into the mucosa, initiating an immunoinflammatory reaction. It is unlikely, however, that bacteria directly cause intestinal ulceration but instead prolong or aggravate the drug-induced damage (Benoni *et al.*, 1985). Indeed, NSAID-induced intestinal ulceration can be significantly reduced by antibiotic treatment. In contrast, bacterial flora are probably not involved in the pathogenesis of gastric injury by NSAIDs since the relatively sterile acidic environment of the stomach minimises contact between bacterial antigens and the gastric mucosa (Rainsford, 1988).

It is worth noting that electron microscopy shows bacterial invasion at the sites of the intestinal mucosa damaged by NSAIDs and the development of the mucosal lesions coincides with a decrease in prostaglandin production (Rainsford, 1986). Therefore, the reduction in prostaglandin levels is probably a manifestation, but not the main causal factor, in the development of NSAID-induced intestinal damage.

A prostaglandin-independent mechanism of NSAID-induced intestinal injury is further substantiated by the finding that a single dose of indomethacin caused a significant reduction in prostaglandin generation within 3h, but it was only after 48h, a time when cyclooxygenase inhibition was declining, that macroscopic intestinal lesions were apparent (Whittle, 1981). These lesions then develop further, despite the return of cyclooxygenase activity to normal.

Redirection of arachidonic acid metabolism

By inhibiting cyclooxygenase, NSAIDs could enhance conversion of arachidonic acid through the 5-lipoxygenase pathway resulting in elevated production of vasoconstrictor peptidoleukotrienes i.e. LTC_4 and LTD_4, plus the generation of an oxyradical during the conversion of 5-HPETE's to HETE's in this pathway (Siegel *et al.*, 1979). Similar diversion to the 12-lipoxygenase pathway by NSAIDs is unlikely as the drugs inhibit peroxidation of 12-HPETE. The leukotrienes and oxygen radicals could be responsible for mucosal cell injury by producing vasoconstriction, stasis, and increased vascular permeability, thereby disturbing gastric mucosal microcirculation. These microvascular disturbances could then lead to hypoxemia, erosions, and deepening of ulcerative lesions (Szabo *et al.*, 1985). The role of leukotrienes in NSAID-induced GI damage is substantiated by the fact that both leukotriene antagonists and inhibitors of 5-lipoxygenase (MK 447, benoxaprofen and BW 755) reduce the gastric and intestinal ulcers caused by indomethacin (Rainsford, 1987). Also, there are less ulcers if both cyclooxygenase and lipoxygenase are inhibited than just cyclooxygenase alone (Rainsford, 1988).

Reactive oxygen species

Reactive oxygen species have one or more unpaired electrons in the outer orbital shell, rendering them chemically reactive. These may damage proteins resulting in enzyme inhibition, cause lipid peroxidation leading to irreversible cellular damage, and react with DNA to cause strand scission and other abnormalities (Lauristen *et al.*, 1989).

These radicals are thought to initiate the tissue destruction in acute and chronic inflammation (Lunec *et al.*, 1981). Ordinarily the host possesses numerous antioxidant enzymes and free radical scavengers in order to protect the tissue against the deleterious effects of reactive oxygen species. However,

in conditions such as rheumatoid arthritis, the inhibitory capacity of these molecules can be overcome in the synovial fluid and degradation of tissue can ensue (Harris *et al.*, 1975).

The gastrointestinal tract is supplied with abundant enzymes necessary for the production of reactive oxygen species. In addition, the attraction of neutrophils to areas of mucosal damage, contributes substantially to the generation of reactive oxygen species via the activation of their NADPH-oxidase (Lauristen *et al.*, 1989) and possibly xanthine oxidase (Rajagopalan, 1980). The involvement of cytotoxic reactive oxygen radicals, namely the superoxide anion O_2^-, the hydrogen peroxide H_2O_2 and the hydroxyl radical OH•, which probably originate from the peroxidative cleavage of 5-HPETE, has been shown to be involved in the pathogenesis of NSAID enteropathy (Del Soldato *et al.*, 1985; and Fisher *et al.*, 1991). This is based on the findings that pretreatment with cysteamine or other free radical scavengers such as catalase, superoxide dismutase, thiourea, and aminopyrine, significantly reduced ethanol- or indomethacin-induced gastric and intestinal mucosal damage in rats.

Adaptive cytoprotection

The ability of the gastric mucosa to increase its resistance to injury when challenged by NSAIDs and other potentially harmful agents such as ethanol, acid, alkali, bile acids, is known as "adaptive cytoprotection" (Robert *et al.*, 1983). It has been proposed that this process involves endogenous prostaglandin synthesis since prostaglandins enhance mucosal defense by enhancing bicarbonate and mucus output, and maintaining mucosal blood flow, plus exogenous prostaglandin therapy effectively mitigates NSAID associated gastric lesions (Konturek *et al.*, 1982). However, mild irritants have not always been shown to stimulate endogenous prostaglandin synthesis,

even though adaptive cytoprotection has occurred. Some agents, at cytoprotective concentrations, have even been shown to depress prostaglandin release (Rees *et al.*, 1984). Indeed, inhibition of PGE_2 by indomethacin did not prevent the cytoprotective changes induced by 20% ethanol against gastric mucosal injury caused by 100% ethanol (Hawkey *et al.*, 1988). Finally, a true 'cyto'-protective effect involving cell membrane stabilisation could not be demonstrated on administration of prostaglandin analogues (Guth *et al.*, 1983). Thus, when assessed microscopically, a mechanism other than stimulation of prostaglandin synthesis appears to be involved in adaptive cytoprotection (Hawkey *et al.*, 1988). There is evidence to suggest that injury to the gastric mucosa is followed by a formation of a gelatinous surface layer, which protects against subsequent injury by the same stimulus (Lacey, 1985).

Neutrophil involvement

The cytokine interleukin-1 has been shown to reduce NSAID injury presumably by affecting neutrophil function (Wallace *et al.*, 1992). The somatostatin analogue, octreotide, also reduces NSAID injury in rats and humans possibly by blocking neutrophil-endothelial interaction (Scheiman *et al.*, 1997).

Nitric oxide involvement

Nitric oxide also appears to maintain epithelial integrity by enhancing mucosal blood flow. Inhibition of nitric oxide synthesis in animal models exacerbates NSAID injury, and nitric oxide donors reduce NSAID toxicity (Wallace *et al.*, 1994a).

1.7 Hypothesis

In the light of all the evidence that NSAID-induced gastrointestinal injury can be caused by mechanisms other than inhibition of prostaglandin synthesis, it has been proposed that the damage is a result of a multistage process of which prostaglandin inhibition plays a part (figure 1.10). Basically, it is suggested that the initial 'topical' biochemical action of NSAIDs (stage 1) would be transformed via a transitional stage of increased intestinal permeability, to generalised macroscopic damage. There follows a brief outline of the hypothesis which will be covered in more detail in subsequent chapters.

On absorption, the NSAID affects the mitochondria causing uncoupling of oxidative phosphorylation (or inhibition of electron transport). As a result, there will be a reduction in adenosine triphosphate (ATP) synthesis together with leakage of calcium (Ca^{2+}) out of the mitochondria. The consequences of this will be: (a) an increase in cytosolic Ca^{2+} with subsequent Ca^{2+} toxicity, (b) generation of reactive oxygen species, (c) a disturbance in Na^{+}/K^{+} and therefore cellular osmotic balance, (d) an increase in adenosine diphosphate (ADP) and adenosine monophosphate (AMP) levels and (d) a loss of control of intracellular tight junctions resulting in an increased intestinal permeability.. The events in this first stage of the damage should be caused by all NSAIDs and occur at any site of absorption, be it the stomach, duodenum, or the small intestine.

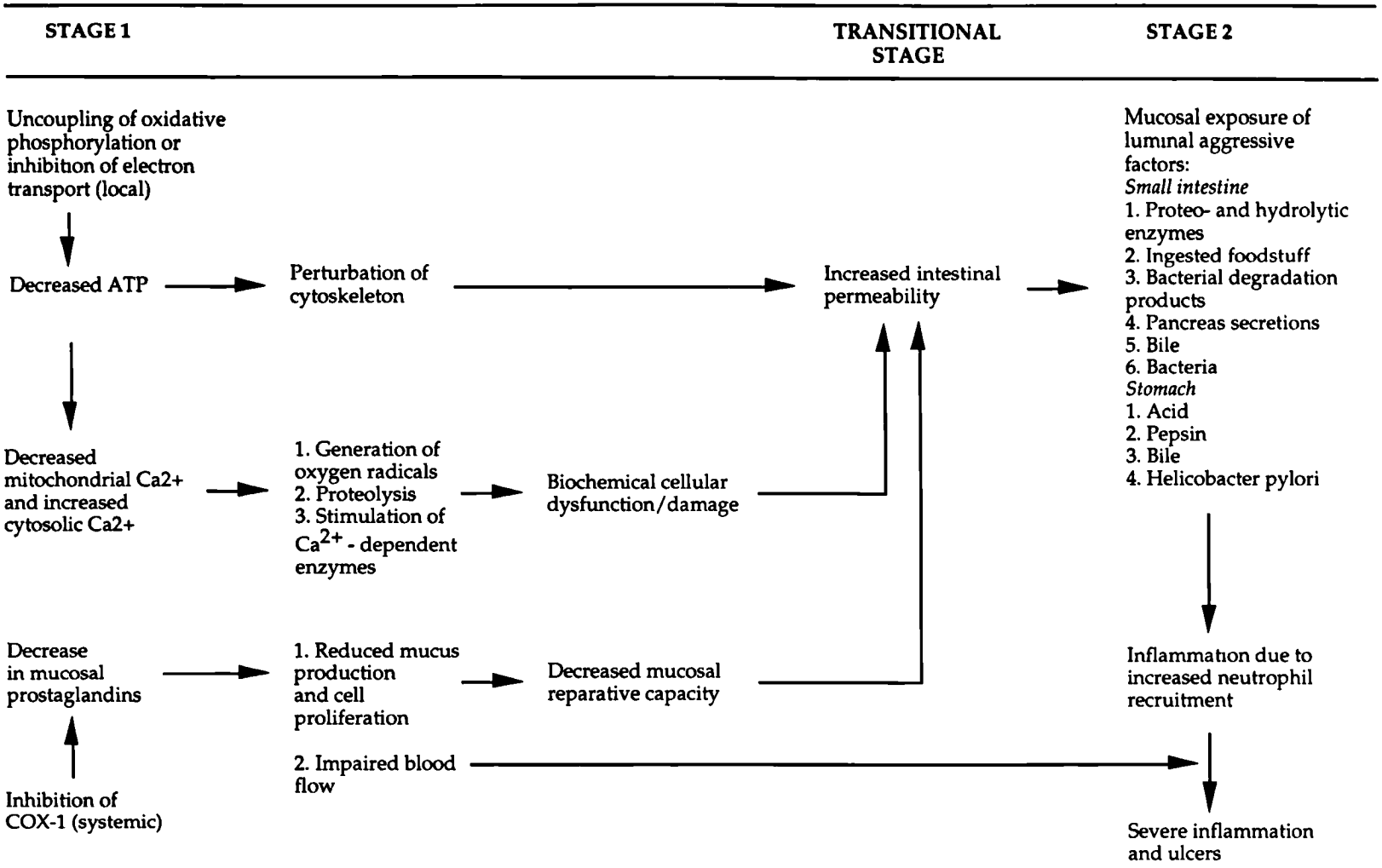

Figure 1.10 The proposed multistage pathway for NSAID damage to the gastrointestinal tract
A schematic outline of NSAID pathogenesis. Adapted from Somasundaram *et al.* (1995).

The increase in intestinal permeability plays an important role in the transitional stage of NSAID damage. The inhibition of cyclooxygenase prolongs rather than initiates the permeability changes by preventing the production of reparative prostaglandins. The increased intestinal permeability permits mucosal exposure to luminal toxins, which results in non-specific tissue inflammation. In a somewhat vicious cycle, this inflammation may act synergistically with the biochemical damage, to further maintain an increase in intestinal permeability.

The second and final stage in the hypothesized mechanism of NSAID-induced GI damage is the macroscopic damage caused by the drugs. The impaired gastric mucosal barrier will enable back-diffusion of acid, pepsin and bile, whereas disruption of the small intestinal barrier exposes the mucosa to bile, digestive enzymes, bacteria and their degradation products.

1.8 Aims of the Thesis

1.8.1 Biochemical Stage

The primary goal of this thesis is to investigate the mitochondrial changes caused by NSAIDs which may preclude to the gastrointestinal damage seen with these drugs. By using the oxygen electrode it will be possible to monitor effects of the compounds on mitochondrial respiration. If the NSAIDs uncouple mitochondrial oxidative phosphorylation then a stimulation of respiration will occur. If the drugs inhibit mitochondrial electron transport then oxygen uptake will decline. By using the artificial electron acceptor ferricyanide, the inhibition, if any, can be localised to particular complex(es) of the electron transport chain. Then using electron paramagnetic resonance

(EPR), changes to individual components of the electron transport chain complexes, in the presence of the NSAIDs, can be viewed. Finally, a measure of mitochondrial ATP production in the presence of the NSAIDs, will further validate whether mitochondrial function has been compromised by these drugs.

1.8.2 Transitional Stage

The transition between the biochemical changes and the macroscopic damage caused by NSAIDs is characterised by increased intestinal permeability. This can be studied in detail by using intestinal cell lines (namely Caco-2 and T84) and measuring effect of the drugs on their transepithelial electrical resistance (TER). A decrease in the TER will reflect an increase in permeability. The TER will be measured firstly by the Ussing chamber and then by the Millicell electrode.

CHAPTER TWO

UNCOUPLING OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION BY NSAIDS

Experimental evidence suggests that the mitochondrion is the main site of early NSAID-induced gastrointestinal damage. Before macroscopic damage is evident, electron microscopy (EM) reveals extensive ballooning, vacuolisation and cristae disruption of the rat gastric (Robins, 1980; and Rainsford, 1975) and intestinal (Somasundaram *et al.*, 1992) mitochondria within only 1 hour of NSAID administration (figure 2.1). At 1 and 6 hours, after NSAID administration there are significant increases in specific mitochondrial marker enzymes, these being succinate dehydrogenase, citrate synthase and cytochrome *c* oxidase. Gross gastrointestinal damage caused by NSAIDs only becomes apparent in rats after 10 or more hours of drug administration.

In order to assess the effect(s) of NSAIDs on mitochondria, it is necessary first to understand the normal structure and function of this organelle.

2.1 Structure of Mitochondria

Mitochondria are subcellular organelles, often located near a source of fuel on which they depend, such as cytoplasmic fat droplets, or near structures such as muscle fibres that require ATP, the major product of their biochemical activity (Lehninger, 1975). Mitochondria take up about 20 percent of the cytoplasmic volume in liver cells and over 50 percent in heart muscle cells.

Mitochondria are generally ellipsoidal, approximately 0.5μm in diameter and 1μm in length. They have two membranes, a smooth outer membrane and an inner membrane folded into cristae which project into the interior matrix (see figure 2.1). Embedded in the phospholipid bilayer of the inner mitochondrial membrane (IMM) are numerous biologically active proteins,

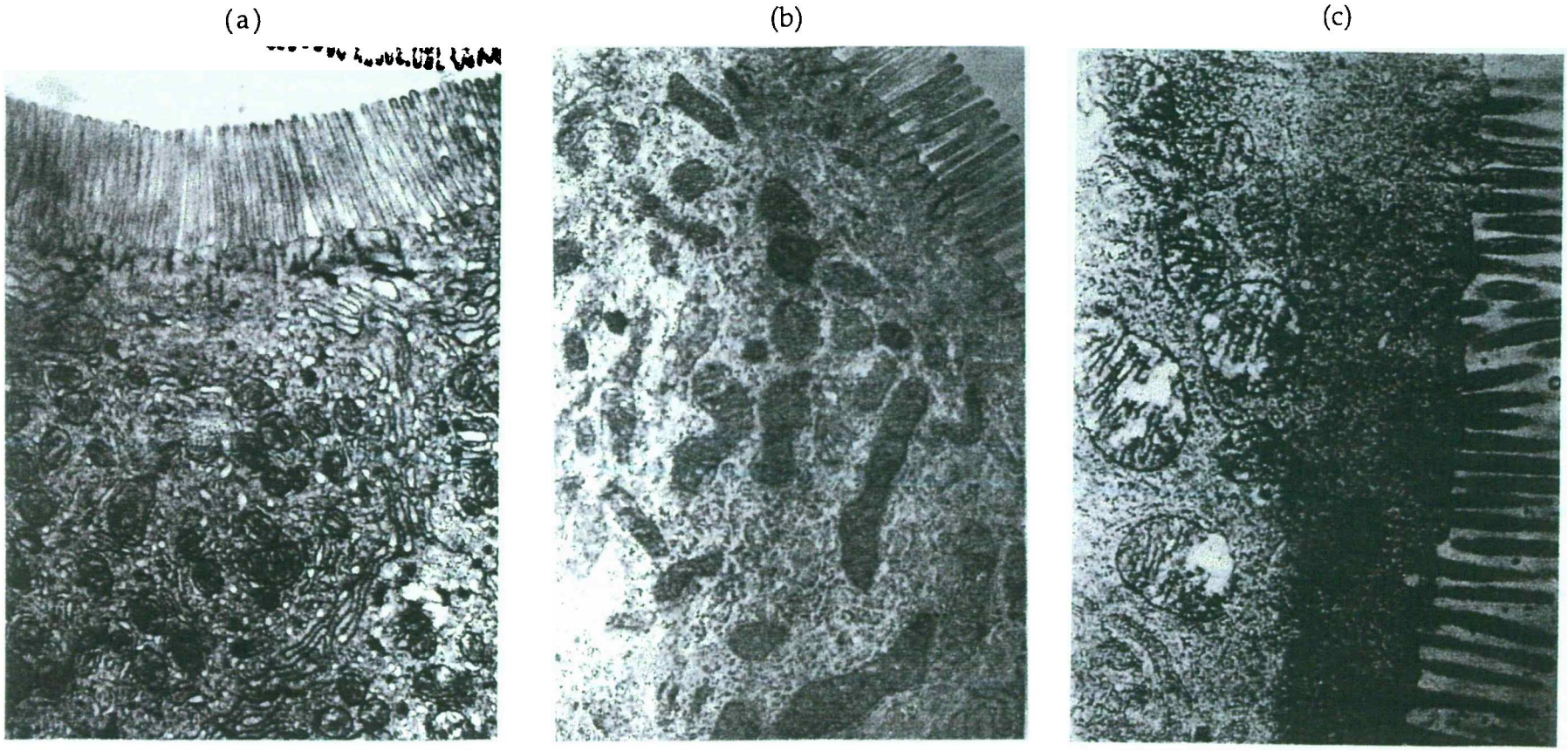

Figure 2.1 Electron micrograph of intestinal mitochondria

EM was carried out on rat enterocytes following 1 hour oral treatment with (a) solvent (control), (b) indomethacin 10mg/kg body weight, or (c) indomethacin 20mg/kg body weight. In (a) the oval-shaped organelles represent normal mitochondria, and the lines within these are the cristae. (b) shows moderate mitochondrial damage seen as mitochondrial elongation. (c) shows severe mitochondrial damage apparent as ballooning and vacuolisation of the mitochondria. Magnification x25,000. Source: electron microscopy unit, King's College, Kensington.

(the IMM is 75% protein by mass). These proteins include electron transfer proteins, enzymes involved in ATP synthesis, certain dehydrogenases, and various metabolite transport proteins such as those for ATP, ADP, pyruvate and Ca^{2+}. The remaining 25% of the IMM comprises of lipids, predominantly phosphatidylcholine (~40%), (~35%), and cardiolipin (~15%). The extent to which the cristae are folded relates to the respiratory activity of the cell, since this increases the surface area of the IMM. Therefore, muscle mitochondria contain a larger number of more densely packed cristae than, say, liver mitochondria. Table 2.1 summarises the enzymes located in the different compartments of rat liver mitochondria.

Inside the inner compartment of the mitochondria is a gel-like protein-rich matrix of <50% water, whose volume and state of organisation changes with alterations in respiratory activity (Voet and Voet, 1995). As well as containing soluble oxidative enzymes (see table 2.1), the matrix also contains DNA, RNA and ribosomes, responsible for generating some mitochondrial proteins. The human mitochondrial DNA is a double helical circle and 60% of its protein-coding capacity is used to specify seven subunits of NADH-coenzyme Q reductase (Complex I) in the IMM (see section 2.2.1). The DNA also encodes a cytochrome reductase subunit, three cytochrome oxidase subunits, and two ATP synthase subunits (Stryer, 1988).

The outer membrane of the mitochondria contains a protein known as porin which forms nonspecific aqueous pores that enable free diffusion of molecules up to 10kDa (Voet and Voet, 1995). The IMM, on the other hand, is impermeable to most ions, metabolites, and low molecular weight compounds and is only freely permeable to O_2, CO_2, and H_2O. Thus, compartmentalisation of metabolic functions occurs between the cytosol and the mitochondrial matrix.

Table 2.1 Location of mitochondrial enzymes

Location of Enzymes
Outer membrane
Monoamine oxidase
NADH dehydrogenase (antimycin-insensitive)
Acyl-CoA synthetases
Phospholipase A_2
Nucleoside diphosphate kinase
Kynurenine 3-monooxygenase
Intermembrane space
Adenylate kinase
Inner membrane
NADH dehydrogenase (antimycin-sensitive)
Iron-sulphur proteins
Cytochromes *b*, *c*, c_1. and aa_3
F_0F_1 ATPase
Succinate dehydrogenase
Carnitine acyltransferase
D-β-hyroxybutyrate dehydrogenase
Matrix
Citrate synthase
Isocitrate dehydrogenase
Fumarase
Malate dehydrogenase
Glutamate dehydrogenase
Aspartate transaminase
Fatty acyl-CoA oxidation enzymes

Adapted from Lehninger (1975).

2.2 Function of Mitochondria

The mitochondrion is known as the "powerhouse" of the cell since it is this organelle which transduces most of the energy derived from respiratory oxidation.

2.2.1 The Respiratory (Electron Transport) Chain

The free energy from the oxidation of fats, amino acids and carbohydrates is made available within the mitochondria as reducing equivalents (H or electrons), mainly carried on NADH. The respiratory chain transports these reducing equivalents to their final reaction with molecular oxygen to form water. Electrons flow through the chain from the more electronegative components to the more electropositive oxygen (figure 2.2). The mitochondria also contains the enzyme systems responsible for producing the reducing equivalents in the first place, i.e. the enzymes of β-oxidation and of the citric acid cycle (Mayes, 1993).

Inhibitors exist which can block electron transport at specific sites in the chain, thereby reducing mitochondrial oxygen consumption. These inhibitors have been used as tools for studying the structure and function of the respiratory chain and will be looked at more closely in chapter 3 .

2.2.2 ATP Generation

The passage of electrons through the protein complexes with progressively greater electron affinity (increasing standard reduction potentials), generates a

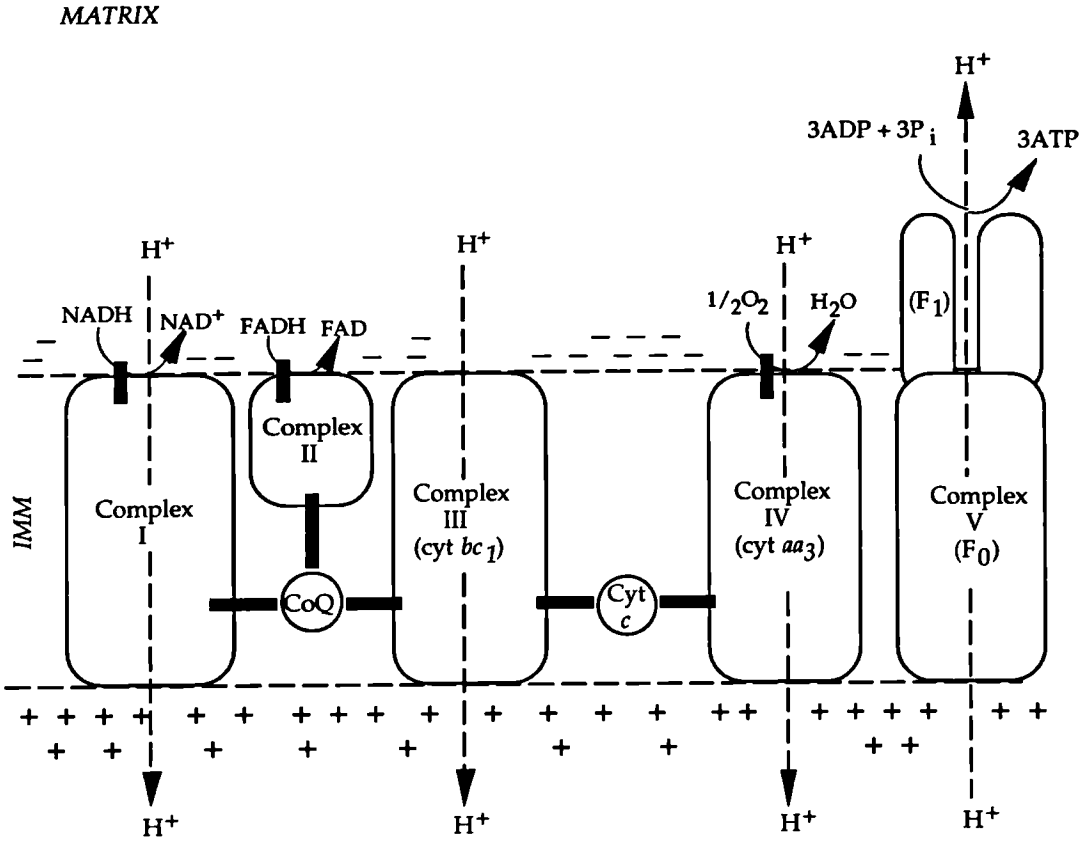

Figure 2.2 The electron transport chain

Electrons are passed from one electron carrier to the next down the electron transport chain, which spans the inner mitochondrial membrane (IMM). This eventually results in the reduction of molecular oxygen to form water. The transport of electrons down the chain (thick lines) causes protons (H^+) to be pumped out of the mitochondrial matrix (dashed lines). The passage of H^+ back into the matrix, through F_1F_0.ATPase (Complex V), enables ATP to be synthesised.

large amount of free energy which is captured to synthesise ATP from ADP and P_i. The process by which mitochondria couples respiration to the generation of ATP is termed "oxidative phosphorylation".

The mechanism by which the energy released from respiration is harnessed to drive ATP synthesis can be explained by the "Chemiosmotic Coupling" model (Mitchell, 1976). The model proposes that the energy from electron transport enables protons to be actively pumped out of the mitochondrial matrix by the respiratory complexes, into the intermembrane space (see figure 2.2). Since the IMM is impermeable to protons, an electrochemical gradient for protons is generated, with a lower pH outside the IMM than inside. In other words, the free energy is used to maintain this proton gradient. When protons do flow back into the matrix down their gradient, it is via the F_1F_0 complex or complex V (figure 2.3). The H^+ passes back into the matrix down a specific proton channel in the F_0 portion of this complex which spans the IMM. The free energy released, as the proton gradient is dissipated, is used to phosphorylate ADP to ATP, and is catalysed by the F_1 component of the complex.

Stoichiometric relationships between the oxidation reactions during electron transport and proton pumping have not been established with certainty (Wrigglesworth, 1997). It is often reported between two and four protons are pumped out of the mitochondria per pair of electrons passing through each respiratory complex. Between 2 and 3 protons are required to synthesise ATP by the F_0F_1ATPase. Other protons are required for various transport systems, such as for the transport of inorganic phosphate into the mitochondria. P/O ratios for oxidation by complexes I, II and III therefore vary according to conditions, but experimental values are usually taken to be 2.6, 1.6 and 1.2

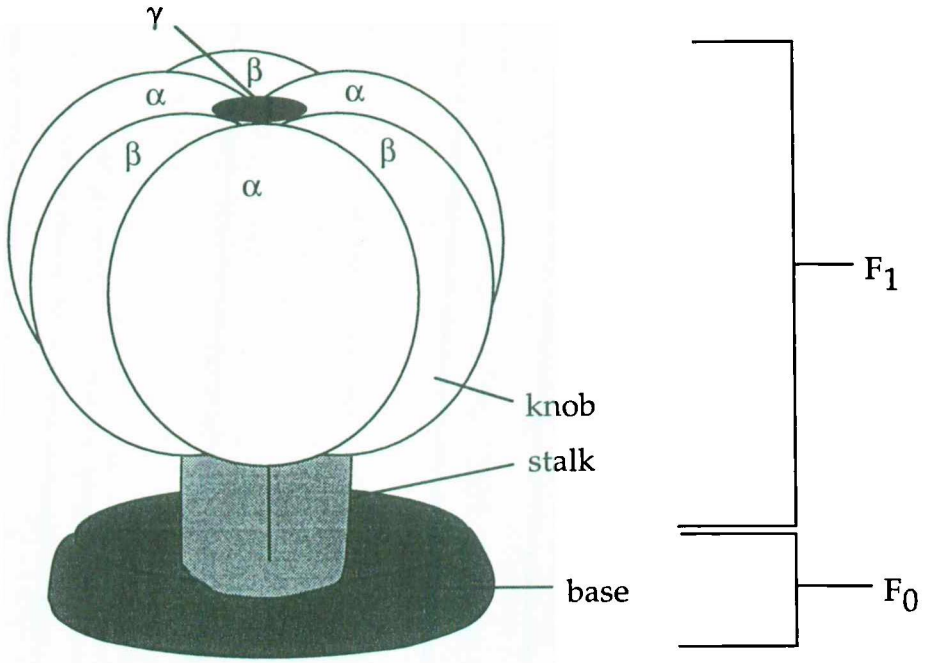

Figure 2.3 Structure of the F_1F_0 complex

The F_1F_0 complex, also known as complex V or ATP synthase, comprises of an F_1 knob, projecting into the mitochondrial matrix, connected by a stalk to the F_0 base. The F_1 knob contains three αβ subunit dimers and one copy each of subunits γ, δ and ε (δ and ε not shown).

respectively for complete oxidation of an electron pair entering into each complex respectively.

2.2.3 Uncoupling of Oxidative Phosphorylation

Electron transport (the oxidation of NADH and $FADH_2$) and phosphorylation (ATP synthesis) are normally tightly coupled. In the resting state when ATP synthesis is minimal, the electrochemical gradient across the IMM continues to build up until it prevents further pumping of protons and inhibits electron transport. A class of compounds have been shown to uncouple these processes and include 2,4-dinitrophenol (DNP) and the 300 times more potent trifluorocarbonylcyanide phenylhydrazone (FCCP). These uncouplers, when added to mitochondria, remove the link between substrate oxidation and ATP synthesis.

Uncouplers are all moderately weak acids with pK_a's in the range 5-7, and therefore will normally be dissociated at intracellular pH. However, when the uncoupler molecule approaches the IMM from the outside, the phenolic hydroxyl group of DNP or the N of FCCP, become protonated due to the lower pH in this region. This protonation makes the molecule more hydrophobic so that it can diffuse into the membrane and pass through down its concentration gradient. Once inside the mitochondrial matrix, the higher pH means that the phenolic hydroxyl group deprotonates (figure 2.4). Thus, the uncoupler acts as a protonophore, transporting H^+ back into the matrix whilst bypassing the F_0 proton channel. Hence, ATP synthesis is prevented by dissipation of the electrochemical gradient. Electron transport can then proceed at a much faster rate due to the lack of inhibition by the electrochemical gradient. This manifests itself as an increase in mitochondrial respiration.

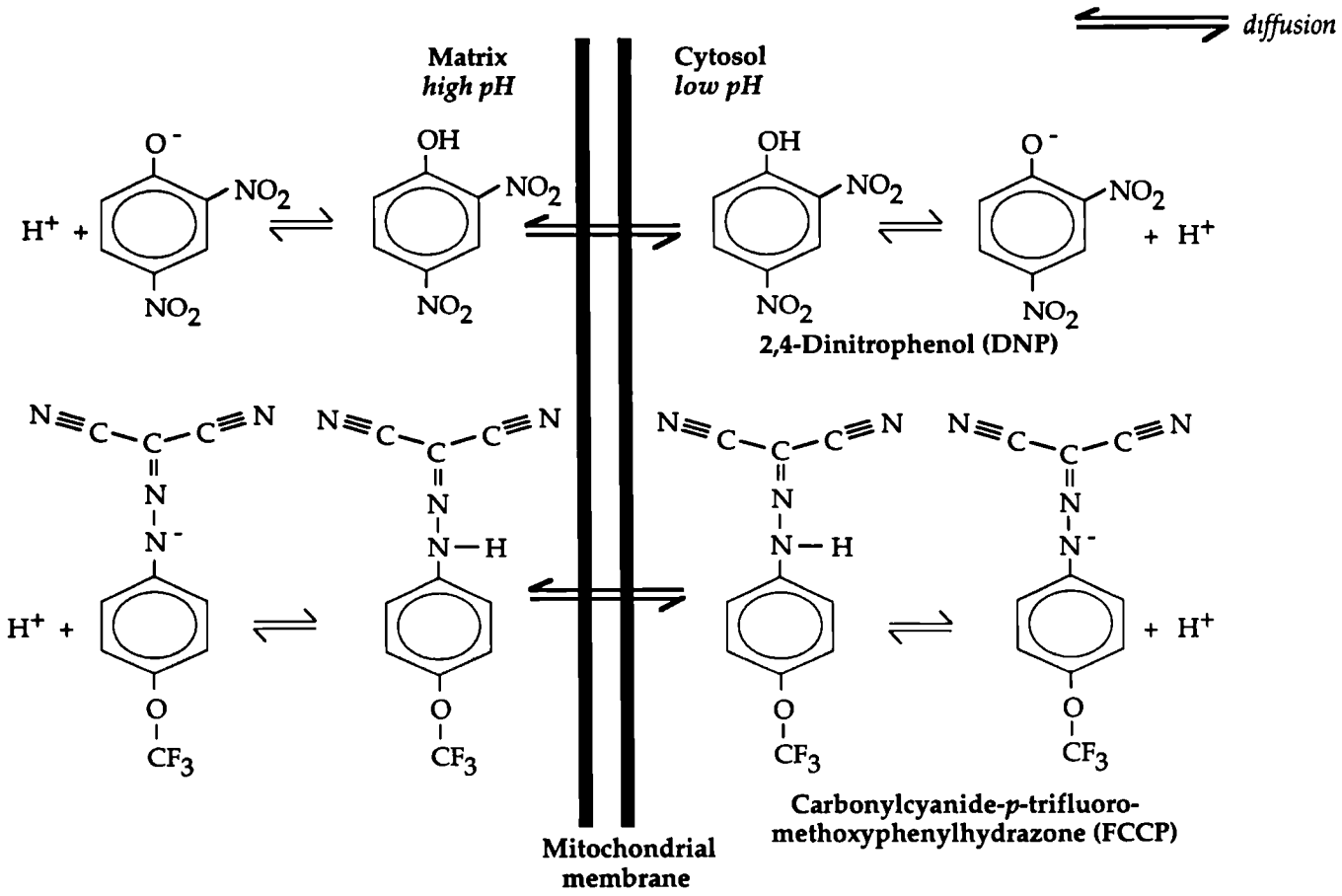

Figure 2.4 Mechanism of action of uncouplers of oxidative phosphorylation

DNP and FCCP uncouple oxidative phosphorylation by transporting protons across the mitochondrial membrane, thereby dissipating the electrochemical proton gradient generated by electron transport. Modified from Voet and Voet (1995).

Another interesting feature of most of the classic uncouplers is that in excessive concentrations (higher than that required to uncouple oxidative phosphorylation), these compounds will inhibit mitochondrial respiration (Kraayenhof and van Dam, 1969), a characteristic not directly related to their uncoupling properties (Wedding *et al.*, 1967). Consequently, these compounds have also been termed "inhibitory uncouplers" (Moreland, 1994). It has been suggested that the uncoupler competes with respiratory substrates (e.g. succinate or glutamate and malate) for penetration into the mitochondrial matrix, and a decrease in the intramitochondrial concentration of these substrates results in an inhibition of mitochondrial respiration (van Dam, 1967; and Quagliariello and Palmieri, 1968). In addition, the diminution in substrate uptake is reversible since it can be overcome by higher substrate concentration (Quagliariello and Palmieri, 1968). This competitive mechanism could explain why a relatively high concentration of uncoupler is required to inhibit respiration. Indeed, at lower uncoupler concentrations, when respiration was still being stimulated, there was no effect of these compounds on substrate uptake (Harris *et al.*, 1967).

An alternative mechanism for uncoupler-induced inhibition of respiration is thought to be mediated by a 31kDa hydrophobic polypeptide, the "uncoupler binding protein" (Hanstein *et al.*, 1979). Binding to this protein of higher uncoupler concentrations than those required for complete uncoupling, has been shown to induce inhibition of respiration (Katre and Wilson, 1978). This protein is thought to reside on the inside of the inner mitochondrial membrane, probably a component of the membrane sector of ATPase i.e. F_0 (Hanstein *et al.*, 1979). However, a later study showed that it was a conformational change of the cytochrome bc_1 complex which elicited inhibition of mitochondrial respiration by uncouplers (Tokutake *et al.*, 1991).

2.3 Effect of NSAIDs on Oxidative Phosphorylation

2.3.1 The Salicylates

As early as 1956 it was reported that salicylates uncouple oxidative phosphorylation in mitochondrial systems (Brody, 1956). Since then, many more studies have also demonstrated the uncoupling of oxidative phosphorylation with aspirin and other salicylates, often apparent as an increase in oxygen consumption (Fishgold *et al.*, 1951; Cochran, 1952; Sproull, 1954; Mackerer *et al.*, 1973; Jorgensen *et al.*, 1976a; Spenney and Bhown, 1977; and Tomoda *et al.*, 1992).

Mechanism of Uncoupling

At first, the exact mechanism of uncoupling by salicylates was not known and it was thought that these compounds, including aspirin, uncoupled by increasing mitochondrial ATPase activity and decreasing phosphate uptake, apparent as an increase in total inorganic phosphate. DNP was shown to be 100 times more potent than salicylate (Miyahara and Karler, 1965; Brody,1956; and Falcone, 1959). Charnock and Opit (1962) suggested that salicylates uncoupled by increasing the permeability of the mitochondrial membrane to ATP, thus stimulating ATPase activity and ATP breakdown. Oxidative phosphorylation was found to be uncoupled in the liver mitochondria of rats fed dietary aspirin (*in vivo*) (Mehlman *et al.*, 1972; and Mackerer *et al*, 1973) and also when aspirin was given *in vitro* (Mehlman *et al.*, 1972). However, only the *in vitro* effects were thought to be attributed to a stimulation of mitochondrial ATPase activity, whereas the mode of uncoupling *in vivo* was perhaps more complex.

It is now known that the uncoupling caused by salicylates is not primarily by stimulation of ATPase activity, as was initially thought. This is because in the presence of oligomycin, an F_1 ATPase inhibitor, there is still an increase in oxygen consumption with salicylate. Instead, salicylic acid acts as a proton ionophore entering the mitochondrial matrix by nonionic diffusion and then dissociating to yield salicylate and H^+ (Gutknecht, 1990). Therefore, the uncoupling caused by salicylates is by a similar mechanism to DNP (Brody, 1956; Jeffrey and Smith, 1959; Whitehouse, 1964a; and Aprille, 1977).

It must be noted that *in vivo* the uncoupling induced by aspirin has been suggested to be due to salicylic acid, to which it is hydrolysed, and not the parent compound itself (Thompkins and Lee, 1969). On absorption from the gastrointestinal tract, aspirin is rapidly hydrolysed ($t_{1/2} \approx 20$min) to form salicylic acid and acetic acid (Needs and Brooks, 1985). The proposed inability of aspirin to uncouple may be because the mitochondrial membrane is relatively impermeable to this drug even in the protonated form, and so there will be very little H^+ uptake into the matrix by aspirin (Gutknecht, 1992).

Therapeutic Significance

Cochran (1954) first speculated that the uncoupling could be of fundamental importance to the therapeutic action of salicylates as antiinflammatory agents since, these drugs, together with phenylbutazone, showed strong antiinflammatory efficacy and also uncoupled oxidative phosphorylation and stimulated respiration. However, this idea was dispelled by the finding that DNP, which strongly uncoupled and increased oxygen consumption, was not antiinflammatory (Adams and Cobb, 1958). Despite the reports that DNP can inhibit both ultraviolet erythema in guinea pigs (Winder *et al.*, 1958) and

dextran/formation oedema in rats (Stenger, 1959), it is still unlikely that the uncoupling activity of DNP is related to these antiinflammatory properties since DNP is highly labile in biological systems and is rapidly metabolised in the liver to 2-amino-4-nitrophenol which has no uncoupling activity (Cross *et al.*, 1949).

Reye's Syndrome

Reye's syndrome is an often fatal syndrome which occurs in children after a prodormal viral illness and is characterised by fatty degeneration of the liver and kidneys, rapid swelling of the brain, seizures, and loss of consciousness (Reye *et al.*, 1963; and Huebi *et al.*, 1987). In patients with Reye's syndrome, the mitochondria are grossly swollen and deformed, with the matrix containing coarsely granular materials, and cristae being few in number, widely spaced, and fragmentary (Thaler *et al.*, 1972). The greater the structural change the more severe the illness. For example, in the comatosed patients the mitochondrial alterations are profound (Lichtenstein *et al.*, 1983). Also the persistent high fever and high overall metabolic rate would suggest uncoupling of mitochondrial oxidative phosphorylation with elevated respiratory rates but decreased ATP formation (Trost and Lemasters, 1996).

It is suggested that aspirin may be associated with outbreaks of the disease since aspirin poisoning has many similarities to Reye's syndrome (You, 1983). Also a decrease in the incidence of outbreaks of Reye's syndrome is paralleled with a decrease in aspirin use in children (Pinsky *et al.*, 1988). Experimental evidence suggests that it is salicylate, the principal metabolite of aspirin, which is involved in the pathogenesis of Reye's syndrome. Salicylate-treated mitochondria and mitochondria from Reye's syndrome patients are ultrastructurally similar. Likewise, the uncoupling and inhibition of

mitochondrial dehydrogenases by salicylate could explain the mitochondrial injury caused by these drugs in Reye's syndrome (You, 1983).

2.3.2 Other NSAIDs

Although the earlier studies were mainly concerned with the salicylates, a wealth of evidence that other NSAIDs also uncouple oxidative phosphorylation, soon emerged. These studies were spearheaded by Whitehouse and associates throughout the 60's and early 70's. Table 2.2 summarises the findings of Whitehouse and other investigators. In the majority of the mentioned studies, the NSAIDs were compared to the classic uncoupler 2,4-dinitrophenol and found to have similar characteristics, albeit to varying degrees.

It has long been suggested that lipid solubility, pK_a, and affinity for protein amino groups are important determinants of the uncoupling activity of NSAIDs (Whitehouse, 1965). Because NSAIDs are generally lipid-soluble weak acids, they themselves will not inhibit specific enzymatic reactions in the mitochondria, but probably act as protonophores in a manner similar to DNP and FCCP (Famaey and Mockel, 1973). In fact, NSAIDs have a chemical structure resembling that of typical uncouplers. They are usually monocarboxylic acids with one or two aromatic rings. Such structures are fairly lipophilic with varying degrees of membrane solubility. Protons are carried across the IMM by a continuous interchange, within the membrane, of the protonated and deprotonated forms of an acidic group of the uncoupler. Thus, an acid dissociable group is a prerequisite for uncoupling activity (Terada *et al.*, 1988). With NSAIDs, this is generally the carboxylic acid side chain with the exception of the piroxicams and phenylbutazone.

Table 2.2 A summary of studies investigating the effects of NSAIDs on mitochondrial function

NSAIDs tested	Properties relating to uncoupling of oxidative phosphorylation	Reference
Phenylbutazone, oxyphenbutazone, salicylic acid, mefenamic acid, flufenamic acid	(i) Stimulate ATPase activity (ii)Inhibition of P_i incorporation into ADP (i.e. ATP biogenesis)	Whitehouse and Haslam, 1962
Indomethacin, phenylbutazone, salicylic acid	(i) Decreased P/O ratio (ii)Inhibition of P_i incorporation into ADP (i.e. ATP biogenesis) without inhibiting oxidative metabolism and respiration	Whitehouse, 1964b
Flufenamic acid, aspirin, ibuprofen	(i) Inhibition of P_i incorporation into ADP (ii) Stimulation of respiration (iii) Stimulation of ATPase activity	Tokumitsu *et al.*, 1977
Indomethacin	(i) Decreased P/O ratio (ii) Stimulation of ATPase activity (iii) Stimulation of respiration	Byczkowski and Korolkiewicz, 1977
Diflunisal, mefenamic acid	(i) Stimulation of ATPase activity (ii) Stimulation of respiration followed by inhibition of this stimulation at higher concentrations	McDougall *et al.*, 1983
Butibufen	(i) Reduction in ATP levels (ii) Increase in oxygen consumption	Míguez *et al.*, 1986
Diflunisal, piroxicam, indomethacin, naproxen, phenylbutazone, sodium diclofenac, potassium diclofenac, tenoxicam, tiaprofenic acid, pirprofen	(i) Increase in oxygen consumption followed by an inhibition of this stimulation at higher concentrations (ii) Concentration-dependent stimulation in ATPase activity.	Baños and Reyes, 1989

Mitochondrial Swelling

Timbrell (1991) has reported that uncouplers evoke contraction of the mitochondria, and that prolonged contraction leads to a deterioration of the inner membrane resulting in high amplitude swelling and eventually membrane rupture. Mitochondrial swelling and disruption of its structure often precedes cell death and necrosis. The NSAIDs indomethacin, flufenamic acid, mefenamic acid, phenylbutazone, oxyphenbutazone, and aspirin have been shown to induce mitochondrial swelling and, initially, it was thought that this occurred at the same concentrations at which they uncoupled oxidative phosphorylation (Famaey, 1973). However, more recently, it was reported higher concentrations of the drugs diclofenac (sodium salt), mefenamic acid, and salicylate were required to uncouple and/or inhibit oxidative phosphorylation than to induce swelling (Mingatto *et al.*, 1996). Therefore, this would suggest that the mitochondrial swelling precedes the uncoupling and/or inhibitory effects of NSAIDs on oxidative phosphorylation. Nevertheless, due to the relatively high concentrations of NSAIDs required to induce such swelling, the relevance to their therapeutic actions remains uncertain.

2.4 Aims of the Studies

In the studies of this thesis, as with most of the studies documented in table 2.2, rat liver mitochondria have been used rather than intestinal mitochondria. This is simply because of the ease with which intact, pure, coupled mitochondria can be prepared in high yields from liver (Rickwood *et al.*, 1987). Coupled intestinal mitochondria are difficult to obtain for routine use. The validity of using rat liver mitochondria as a surrogate for studying uncoupling is, however, consistent with the universality of the chemiosmotic

coupling mechanism between electron transfer and oxidative phosphorylation (McLaughlin and Dilger, 1980).

Oxidative phosphorylation and the effect of agents which uncouple it, can be assessed experimentally by measuring the rate at which a suspension of mitochondria consumes oxygen. This is most conveniently done using an oxygen electrode which can rapidly and continuously assay the respiratory activity of small samples of mitochondria. When electron transport is uncoupled from ATP synthesis, oxygen uptake proceeds at a higher rate even in the absence of added ADP. Uncoupling can be achieved chemically, as noted earlier, by compounds such as FCCP (Mathews and van Holde, 1996).

There follows a brief outline of various studies carried out on the effects of NSAIDs on oxidative phosphorylation.

Study 1

The aim was to study the effect of the NSAIDs indomethacin, aspirin, naproxen, piroxicam and ketoprofen on mitochondrial oxygen uptake. The control was the analgesic paracetamol which has very little or no antiinflammatory properties. The effect of these drugs were compared to the classic uncoupler FCCP.

Study 2

To investigate if the acidic pro-NSAIDs sulindac and fenbufen had any uncoupling effects on mitochondrial respiration. Both fenbufen and sulindac need to be metabolised *in vivo* to exert antiinflammatory activity. It is the sulphide metabolite of sulindac (Duggan *et al.*, 1977) and the biphenylacetic acid metabolite of fenbufen (Tolman and Partridge, 1975) which are important for the drug's ability to inhibit cyclooxygenase. Likewise, the effect of the

nonacidic prodrug nabumetone was studied and compared to its active metabolite 6-methoxy-2-naphthylacetic acid (6-MNA).

The acid-dissociable group is an essential requirement for a compound to be able to uncouple. Analogues of uncouplers, where the acid-dissociable group has been replaced by a non-dissociable group, have no uncoupling activity (Terada *et al.*, 1988). Therefore, one would expect that the compounds fenbufen, sulindac and 6-MNA to uncouple mitochondria *in vitro* since they are acidic and nabumetone not to uncouple.

Study 3

To study the effect of the NSAID flurbiprofen and its nitroxylbutyl ester derivative NO-flurbiprofen. It has been reported that NO-NSAIDs (including NO-flurbiprofen) cause little or no gastrointestinal injury, whilst being equally as effective as an antiinflammatory agent or cyclooxygenase inhibitor as the native NSAID (Wallace *et al.*, 1994a,b). Moreover, NO-flurbiprofen has been shown to accelerate the healing of experimental gastric ulcers (Elliot *et al.*, 1995) and to be associated with significantly less intestinal ulceration than flurbiprofen (Somasundaram *et al.*, 1997). It is postulated that this is due to the release of the nitric oxide (NO) group during drug absorption (Davies *et al.*, 1997; and Reuter *et al.*, 1994, 1997). This is substantiated by the findings that NO or other drugs that generate NO (e.g. sodium nitroprusside or glyceryl trinitrate) reduce the severity of experimental gastric mucosal injury (MacNaughton *et al.*, 1989; and Kitagawa *et al.*, 1990).

NO is an inorganic gaseous free radical, shown to possess a variety of biological functions, including regulation of vascular tone, platelet aggregation, leukocyte adhesion, and immune host defense (Moncada *et al.*, 1991). Abnormalities of NO production may play a central role in the

pathogenesis of many disease processes. For example, inhibitors of NO synthesis appear to exacerbate gastric mucosal injury induced by indomethacin by reducing mucosal blood flow, causing neutrophil adherence to the vascular endothelium (Pique *et al.*, 1989; and Kubes *et al.*, 1991).

It is possible, therefore, that NO-flurbiprofen will not uncouple isolated mitochondria, but flurbiprofen will.

The stability of NO-NSAIDs as supplied is stated in the manufacturer's data on file as being greater than one year without deterioration. Whether the NO moiety may be released *in vitro*, however, has not been assessed.

Study 4

To investigate if the orally active, synthetic prostaglandin E_1 methyl ester analogue, misoprostol, could prevent the uncoupling induced by indomethacin in isolated mitochondria. Conventional antiulcer therapies, such as H_2 receptor antagonists, antacids, and sulcrafate, are ineffective in preventing NSAID-induced ulceration and so are not approved for this use by the US Food and Drug Administration (FDA) (Wallace, 1992). Prostaglandin analogues, on the other hand, have been approved for the treatment of NSAID-induced gastropathy.

In clinical trials, the prostaglandin misoprostol has been shown to reduce NSAID-induced gastroduodenal injury both in the short and long term (Silverstein *et al.*, 1995). Similar findings have been demonstrated in the rat model (Davies *et al.*, 1994). However, it is not clear if the protective effect of misoprostol is a mitochondrial-, prostaglandin- or vascular-mediated effect.

In order to assess the *in vivo* effects of indomethacin in the presence and absence of misoprostol on the mitochondria, electron microscopy (EM) studies was performed. 4 rats were given (1) control (solvent only), (2) indomethacin 10 mg/kg body weight, (3) misoprostol 60 μg/kg body weight or (4) indomethacin and misoprostol.

Study 5

To study the effect of a mixture of glucose and citrate on indomethacin-induced mitochondrial uncoupling. In the whole animal, a similar glucose-citrate formulation (containing sodium acid citrate) of indomethacin (in a ratio of 15:15:1) has been shown to protect the gastrointestinal tract from the deleterious effects of indomethacin (Rainsford and Whitehouse, 1980; and Davies *et al.*, 1994). In humans, administration of 15mg glucose and 15mg sodium citrate to each mg of indomethacin, prevented the expected increase in small intestinal permeability caused by indomethacin in the short-term (Bjarnason *et al.*, 1992).

Likewise, a glucose-citrate-azapropazone mixture reduced the gastric mucosal damage in rats and faecal red cell loss in human volunteers, when compared to subjects treated with azapropazone alone (Rainsford *et al.*, 1991). Though glucose and citrate reduced the ulcerogenic properties of azapropazone, these nutrients did not modify the ability of azapropazone to relieve pain and inflammation. Therefore, it would appear that such an NSAID formulation has great clinical potential as one which produces less gastrointestinal side effects. However, long-term safety studies on these compounds have not been performed.

In the experiments of this study, a glucose-citrate-indomethacin mixture (Na^+ salts) was made up in a ratio of 15:15:1 in accordance with the study carried

out by Bjarnason *et al.* (1992). The effects of this mixture on indomethacin-induced uncoupling was investigated *in vitro* in isolated mitochondria.

For *in vivo* assessment, rats were orally administered (1) solvent only, (2) 450 mg/kg body weight glucose and 450 mg/kg body weight citrate, (3) 30 mg/kg body weight indomethacin, or (4) indomethacin with glucose and citrate (1:15:15). The intestinal mitochondria were then viewed by EM.

2.5 Materials and Methods

2.5.1 Animals

Throughout these studies male Sprague-Dawley rats (Charles River Laboratories), 6-8 weeks old and weighing 200-250 g were used.

2.5.2 Materials

Reagents

The following were purchased from Sigma Chemical Company Ltd., Dorset, England: the buffer ingredients 3-[N-morpholino]propanesulfonic acid (MOPS), D-mannitol, fatty acid-free bovine serum albumin (BSA); substrates sodium (Na) succinate, Na_2 adenosine diphosphate (ADP); uncoupler carbonyl-cyanide-p-trifluoromethoxyphenyl hydrazone (FCCP); drugs indomethacin, aspirin, naproxen, piroxicam, ketoprofen, sulindac, fenbufen, flurbiprofen, paracetamol.

British Drug House (BDH) Ltd., Poole, England, supplied the buffer constituents sucrose, Na_2 ethylenediamine tetraacetic acid (EDTA), potassium

chloride (KCl), tris[hydroxymethyl]aminomethane (tris), magnesium chloride ($MgCl_2$), potassium dihydrogen orthophosphate (KH_2PO_4); the substrates glucose and sodium citrate; sodium hydroxide (NaOH) and concentrated hydrochloric acid (HCl) required for neutralisation; the solvents dimethyl sulphoxide (DMSO) and ethanol; and the reducing agent sodium dithionite ($Na_2S_2O_4$).

Misoprostol was obtained as a powder from Searle, High Wycombe, U.K. Nitrobutyl-flurbiprofen was supplied by Nicox SA, Paris, France.

Instruments

Homogenisation of tissue was achieved by using a glass-Teflon motorised Potter-Elvejhem homogeniser at a fixed speed. Centrifugation was performed on a Beckman centrifuge with the JA-20 fixed rotor. Oxygen consumption measurements were done using a Clarke-type oxygen electrode fitted with a thermostatted Plexiglass reaction chamber (Rank Brothers, Cambridge, U.K.). The electrode was connected to a chart recorder running at a speed of 1cm/minute. All glassware and centrifuge tubes were kept cool (4°C) prior to use, and buffers and solutions were kept on ice throughout the experiment. In order to prevent electrode poisoning and compounds attaching to the chamber wall, the electrode was cleaned and the chamber soaked in ethanol overnight then rinsed thoroughly, before each experiment.

2.5.3 Methods

Solubulisation of the Compounds

Succinate and ADP were prepared as 1M and 20mM stock solutions respectively by dissolving in distilled water. The uncoupler FCCP was prepared as a 1mM stock solution in 100% ethanol. Stock solution

concentrations for the drugs were as follows: 50mM indomethacin, 100mM aspirin, 100mM and 250mM paracetamol, 1M naproxen and 2mg/ml misoprostol. Indomethacin, aspirin and paracetamol were prepared in 10% DMSO and the pH adjusted to 7.4 with 1M NaOH. (The conventional method of dissolving these drugs in sodium bicarbonate was not used as the high pH of this solution would itself have affected mitochondrial respiration (Tobin *et al.*, 1972)). Naproxen and misoprostol were soluble in water. Control experiments containing either 10% DMSO or water, showed that these solvents by themselves did not affect mitochondrial respiration at the volumes used in the present studies.

Isolation of Rat Liver Mitochondria

This was essentially the same as Schneider and Hogeboom (1950) with a few modifications. The rat was killed by cervical dislocation and the liver rapidly dissected out. Using scissors, the tissue was chopped up in ice-cold saline (0.9%) to approximately $1cm^3$ pieces and the saline decanted off. The tissue pieces were washed once more with fresh saline, which was again decanted, in order to remove any excess blood.

The chopped liver was placed in 50ml homogenising buffer consisting of 75 mM sucrose, 225 mM mannitol, 10 mM MOPS, 1mM EDTA, 5mg/ml BSA, pH adjusted to 7.4 with 1 M NaOH. (BSA was included to remove the fatty acids found in isolated mitochondria which may uncouple the mitochondria (Matsuoka and Nakamura, 1979; and Soboll *et al.*, 1984)). The liver was then homogenised by passing the teflon homogeniser up and down the tube 6 times.

The homogenised mixture was then divided equally into two precooled 50ml polythene centrifuge tubes, each made up to approximately 30 ml with

homogenising buffer, then centrifuged at 500 g for 10 minutes. This sediments the heavier particles, such as any unlysed cells, nuclei and large membrane fragments. The supernatant was then carefully decanted into fresh tubes, made up to 30 ml and centrifuged for 10 minutes at 12,000 g. A crude mitochondrial pellet is sedimented but needs to be 'washed' as it contains other components such as lysosomes, peroxisomes and membrane fragments. Therefore, the supernatant was removed, and the pellet resuspended in 30 ml buffer, then this mixture again spun at 12,000 g for 10 minutes. The supernatant was discarded and the pellet resuspended in a minimal volume of homogenising buffer. This yields a relatively pure mitochondrial preparation (Rickwood *et al.*, 1987), and takes no more than 1½ hours.

Protein Determination

This was carried out using Pierce's protein assay kit (Pierce, Illinois, U.S.A.), with BSA being used as the protein standard. Mitochondrial protein concentrations could then be measured spectrometrically at an absorbance of 562nm.

Measurement of Oxygen Consumption

Electrode set-up

Mitochondrial respiration, hence the functioning of the electron transport chain, was measured using a Clarke-type oxygen electrode (Clarke, 1956) as first described by Chance and Williams (1956). The oxygen electrode consists of a silver/silver chloride reference anode surrounding a platinum cathode (Rickwood *et al.*, 1987; and figure 2.5). These electrodes are immersed in saturated KCl solution and separated from the main chamber by a thin Teflon membrane which is permeable to oxygen but also prevents electrode poisoning.

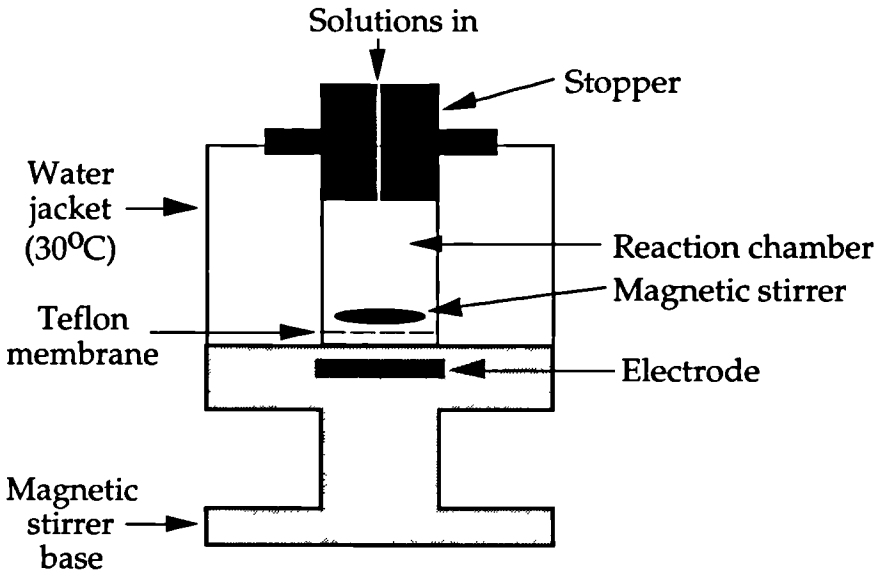

Figure 2.5 The oxygen electrode

This Clarke-type oxygen electrode enables direct measurement of the oxygen content of solutions within its reaction chamber. Problems of measurement can arise by uneven stirring and by back-diffusion of oxygen through the Perspex walls of the chamber.

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At the platinum cathode, oxygen molecules are reduced by electrons to water:

$$4H^+ + 4e^- + O_2 \longrightarrow 2H_2O$$

whilst the chloride anions migrate to the silver anode, releasing electrons:

$$4Ag + 4Cl^- \longrightarrow 4AgCl + 4e^-$$

The transfer of electrons from the cathode to the anode creates a current between the two electrodes which can be measured in an external circuit. With the electrodes polarised at 0.6 volts, the current generated is proportional to the partial pressure of oxygen in the sample. This current is also very temperature-sensitive and, therefore, it is important to operate the electrode at a constant temperature by means of a water temperature jacket.

The oxygen electrode buffer comprising of 150 mM sucrose, 20 mM KCl, 10 mM tris, 5 mM $MgCl_2.6H_2O$, 1 mM KH_2PO_4, pH 7.4 with concentrated HCl, is vigorously stirred with a magnetic stirrer. This ensures an equilibrium between the oxygen dissolved in the solution and the gas diffusing through the Teflon membrane of the electrode.

The electrodes are enclosed in a small chamber of 1ml capacity which is sealed by a close fitting cap, except for a tiny aperture for addition of solutions, to minimise the stirring in of oxygen from the surrounding air.

Calibration of the oxygen electrode

1. 1ml distilled water was added to the reaction vessel and stirred in order to achieve maximum saturation with atmospheric oxygen at the measurement temperature allowed 30°C and then the stopper inserted.

2. The pen of the chart recorder can be adjusted to full deflection using an appropriate choice of sensitivity. This represents 250μM oxygen (i.e. atmospheric oxygen dissolved in distilled water at 30°C).

3. On removal of the stopper, a few crystals of sodium dithionite (a strong reducing agent) is added to the reaction chamber to purge the dissolved oxygen, and the stopper reinserted. The pen rapidly drops to the bottom of the chart paper and is adjusted to a zero mark, indicating 0% oxygen in the mixture. Thus, two concentrations of oxygen, 0 and 250µM, are known from which other concentrations can be determined assuming a linear response of the electrode.

4. The chamber is washed out thoroughly with distilled water before proceeding with any experiments, as the Plexiglass (plastic) vessel wall has an avidity for dithionite (together with lipid soluble compounds), so any carry-over to the next experiment may give spurious results (Estabrook, 1967).

Experimental procedure

1ml of oxygen electrode buffer was added to the reaction chamber and allowed to equilibriate with atmospheric oxygen for approximately 5 minutes, whilst being stirred at 30°C.

On insertion of the stopper, mitochondria, substrates, drugs etc., were added The general sequence of addition was as follows, for (a) the measurement of phosphate/oxygen (P/O) and respiratory control (RCR) ratios: 100µl isolated mitochondria, 1mM succinate, and 0.2mM ADP; and (b) the uncoupling experiments: mitochondria, succinate, FCCP or drug, then ADP. Each different concentration of drug was added to a fresh sample of mitochondria. Solutions added never exceeded 200µl. After every addition a period of approximately 1-2 minutes was allowed to elapse in order to observe measurable rates for oxygen uptake on the chart recorder.

Succinate was used as the substrate in these experiments because it gives a good rate of respiration against which uncoupling can be compared. The fact that succinate donates electrons specifically to complex II of the mitochondrial respiratory chain was of no significance to the present experiments since uncoupling is not substrate-dependent (Terada, 1981).

Measurement of P/O and respiratory control ratios

Respiratory control is generally regarded as an excellent index for the integrity of isolated mitochondria (Hatefi *et al.*, 1961). When 'tightly coupled' mitochondria are added to an isotonic buffer a relatively slow rate of oxygen consumption is measured with the oxygen electrode, caused by oxidation of endogenous substrates. Addition of an oxidisable substrate such as succinate increases the respiration rate. On addition of ADP, there is an immediate and further increase in the rate of oxygen uptake, known as state 3 respiration (Chance and Williams, 1956). State 3 respiration reflects the conversion of ADP to ATP and its duration is dependent on the amount of ADP added. State 3 respiration will persist until all the ADP has been phosphorylated to ATP. The rate of respiration then slows down again, and this is known as state 4 respiration (figure 2.6).

Since the concentration of oxygen utilised is proportional to the amount of ADP phosphorylated to ATP, the ADP/O (or P/O) ratio can be directly measured from the oxygen electrode traces. With succinate as the substrate, the P/O ratio should be close to 1.6 for coupled rat liver mitochondria.

$$\text{The RCR} = \frac{\text{rate of oxygen uptake in state 3}}{\text{rate of oxygen uptake in state 4}}$$

The RCR is normally around 4-6 for intact isolated mitochondria.

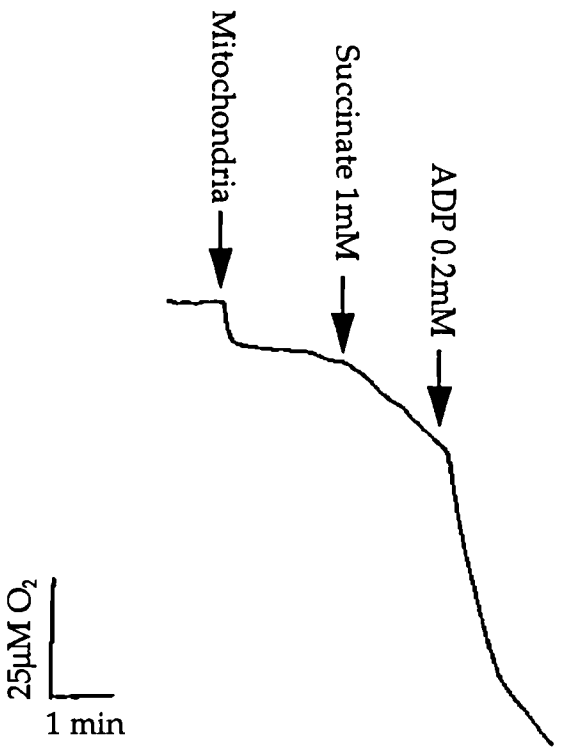


Figure 2.6 Oxygen electrode trace demonstrating P/O and RCR measurement

This is a typical trace of 'tightly coupled' mitochondria, obtained prior to starting each of the experiments. The concentrations given are the final concentrations in the electrode. The P/O ratio is the nanomoles of ADP added, divided by the nanograms atoms of total oxygen consumed from the start of state 3 respiration to its end. The RCR is calculated by dividing the rate of oxygen uptake in state 3 by the rate of oxygen uptake in state 4 (see main text). If by measuring the P/O and RC ratios, the mitochondria were found not to be 'tightly coupled', then the mitochondria were not used.

Therefore, before carrying out experiments with freshly isolated mitochondria, it is essential to ascertain that the mitochondria are tightly coupled i.e. their structure is intact, by measuring the P/O and RCR ratios (Mathews and van Holde, 1996). Incomplete phosphorylation of ADP or partial hydrolysis of ATP during the state 3 period, caused by damaged uncoupled mitochondria, can be observed as a decrease in the expected P/O or RCR values.

Electron microscopy studies

Two hours after drug administration an abdominal incision was made in the rat under anaesthesia (hypnovail-hypnonorm), and the stomach was opened. A catheter was placed in the second part of the duodenum and the whole of the intestine flushed, avoiding distension, with a solution of gluteraldehyde (3% v/v) in 0.1M sodium phosphate buffer pH 7.4. A 1cm length of jejunum (10cm distal to the ligament of Trietz) was then excised, and placed in gluteraldehyde for 3 days.

Sections from this tissue were made to 3μm width by an Ultratome-Richart Ultracut-E, stained in Reynold's lead citrate medium for 10 minutes and viewed with a Joel 1200cm electron microscope in transmission mode. In particular, regions with well orientated cuts containing at least 10 consecutive enterocytes for analysis, were studied.

For quantitative assessment, a total of 100 enterocytes from each animal was examined for mitochondrial changes. Mild changes were only accepted as results if they occurred in a cluster of 5 or more adjacent mitochondria. Definition of mild mitochondrial abnormality is elongation with mild swelling of the mitochondria. Severe abnormalities include distension, ballooning, vacuolisation, and disruption of the outer and inner membranes

of the mitochondria. All the samples were coded so that the morphological assessment was performed with no knowledge of treatment.

2.6 Results

Study 1: In vitro effects of NSAIDs on mitochondrial respiration

The NSAIDs indomethacin, aspirin, naproxen, piroxicam and ketoprofen all stimulated oxygen uptake of mitochondria over the concentration range 0 to 3.75mM. Representative traces of the effects of the NSAIDs on mitochondrial function are shown in figures 2.7a, b and c.

When the effect of the NSAIDs is expressed as a percentage of control,

i.e.

$$\frac{\text{rate of oxygen uptake in the presence of drug}}{\text{rate of oxygen uptake in the absence of drug}} \times 100$$

over the concentration range of drug used, then a stimulation in oxygen uptake can be seen by all the NSAIDs, followed by an inhibition of this stimulation at higher concentrations of the drugs (figures 2.8a and b). These figures show that the most potent uncoupler amongst the NSAIDs (defined as the least drug concentration to achieve maximum stimulation of oxygen uptake) was indomethacin, requiring only 0.25 mM. However, among the NSAIDs, it was aspirin which stimulated mitochondrial respiration to the greatest extent (294%).

The potency of and extent of respiration brought about by the classic uncoupler FCCP was far greater than any of the other drugs. FCCP enhanced mitochondrial respiration to more than 600% at only 4×10^{-4} mM. Table 2.3 summarises the concentrations of the various NSAIDs required to give maximal stimulation of oxygen uptake.

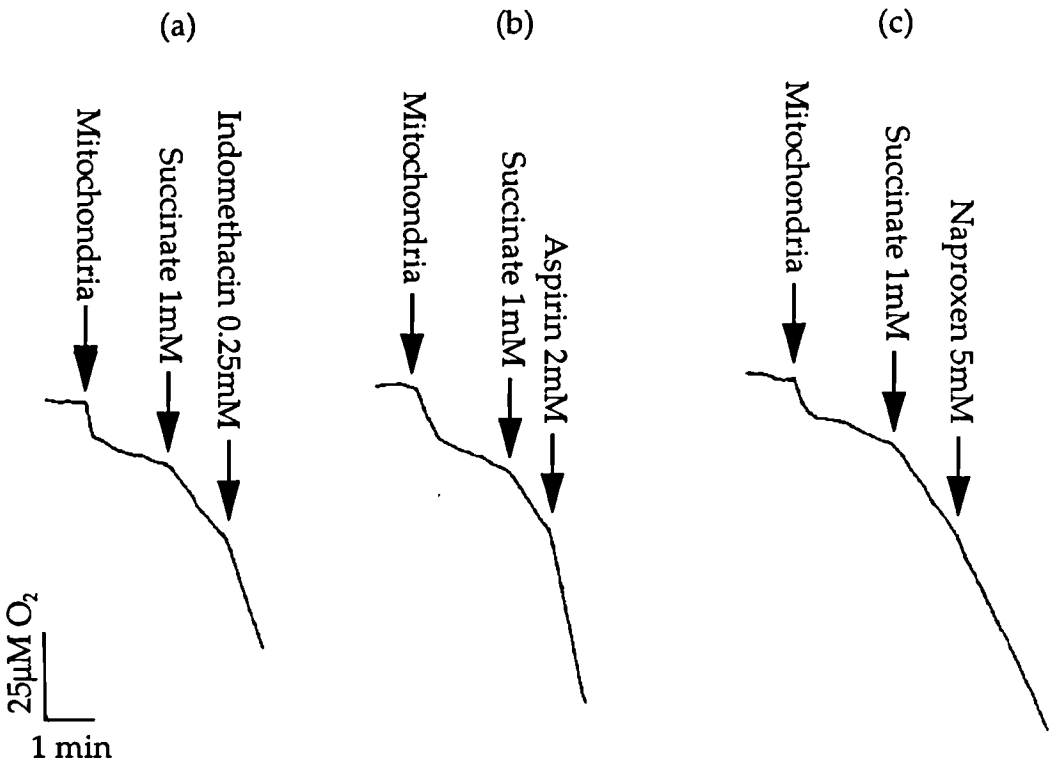


Figure 2.7 Oxygen electrode traces demonstrating uncoupling of oxidative phosphorylation by NSAIDs

To mitochondria (1.2 to 4.2mg protein) was added succinate followed by the NSAIDs (a) indomethacin, (b) aspirin and (c) naproxen, to the final concentrations indicated. Mitochondrial oxygen uptake was monitored using an oxygen electrode (see main text for details).

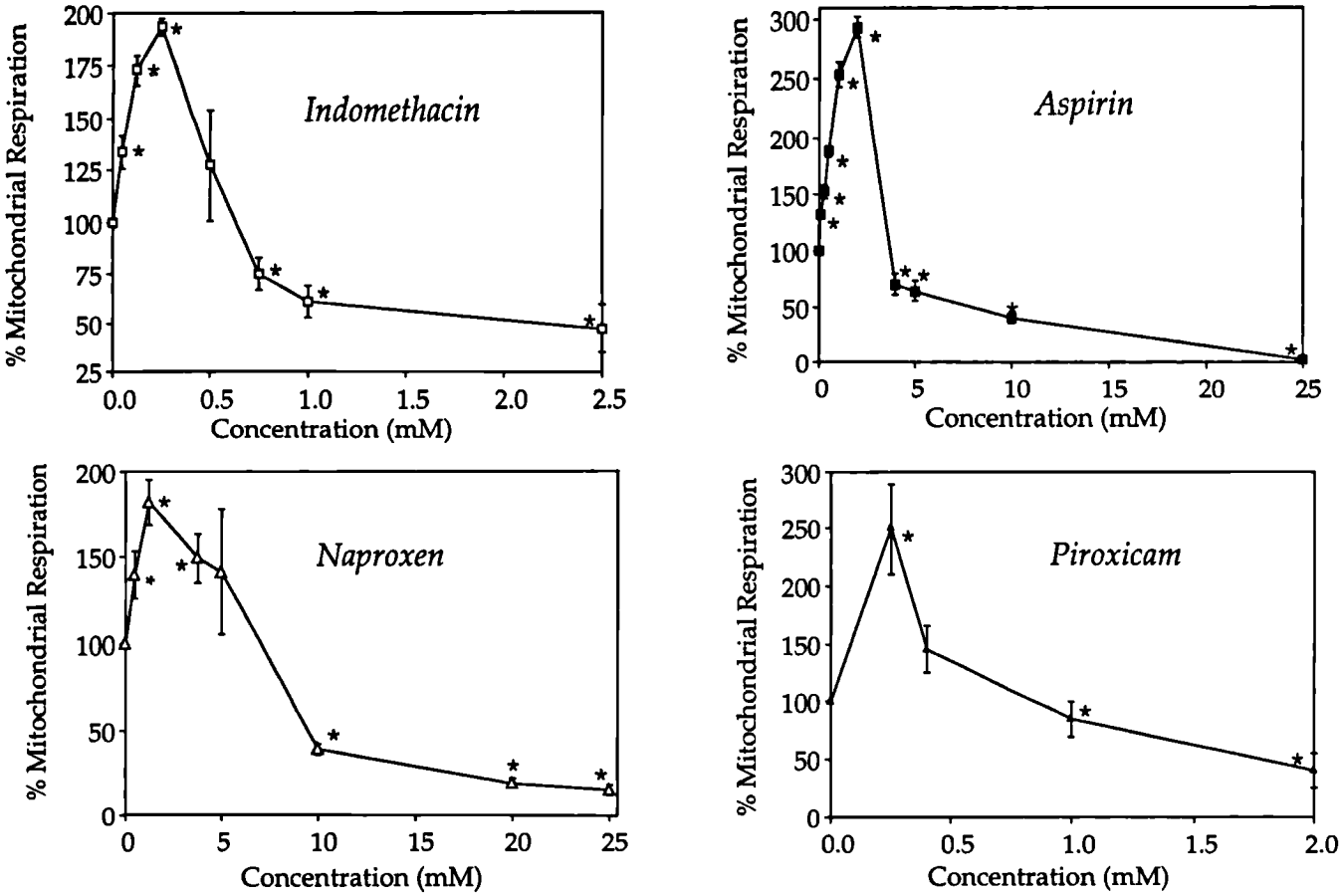


Figure 2.8a Effect of drugs on mitochondrial respiration

To 1.2-4.2mg mitochondria was added 1mM succinate, then drug to the final concentrations indicated. Mitochondrial respiration was monitored at 30°C using an oxygen electrode. Data are the mean ± SEM of 3 experiments (separate liver preparations). * Significantly different from control, $P < 0.05$, Student's t-test.

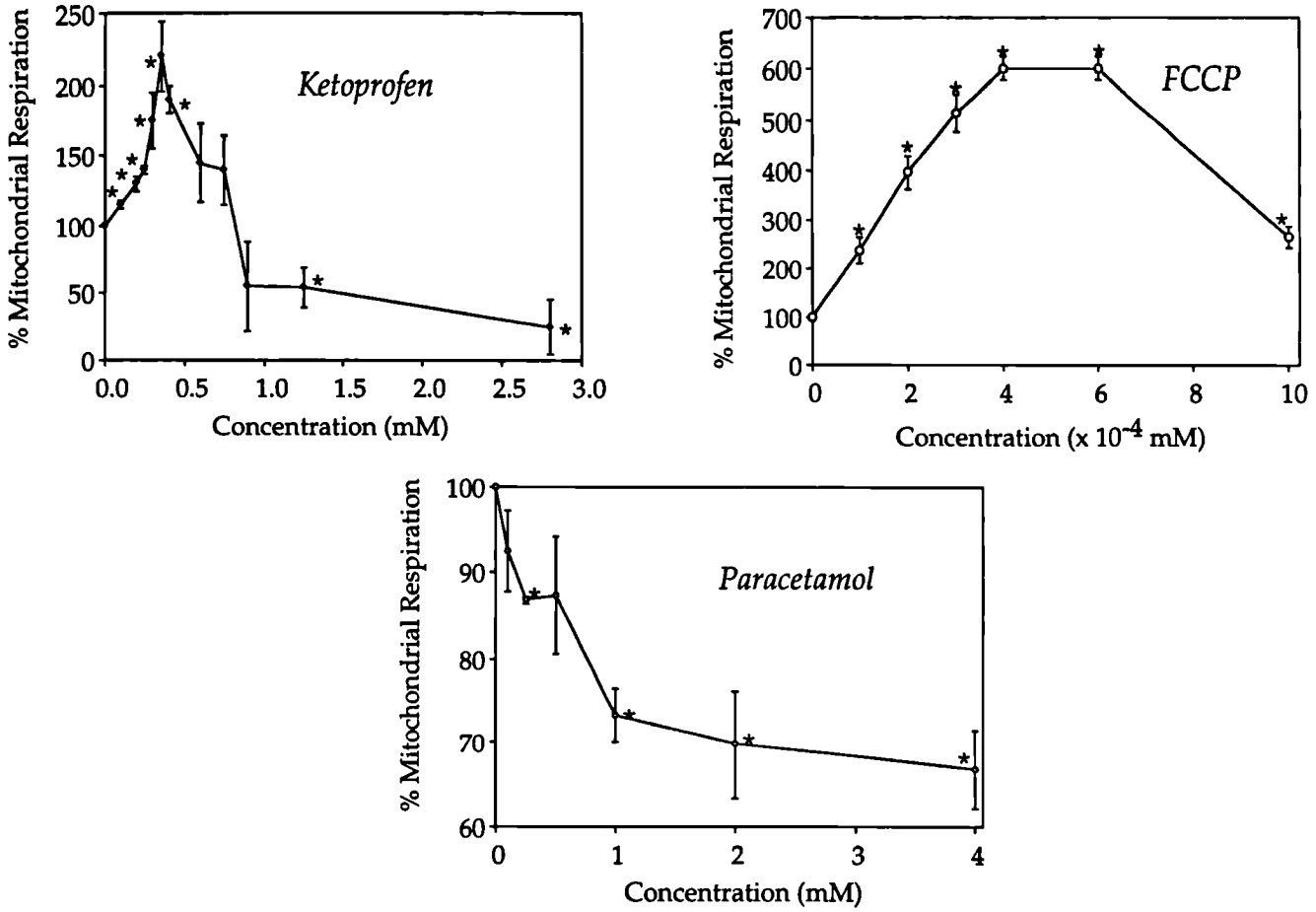


Figure 2.8b Effect of drugs on mitochondrial respiration

To 1.2-4.2mg mitochondria was added 1mM succinate, then drug to the final concentrations indicated. Mitochondrial respiration was monitored at 30°C using an oxygen electrode. Data are the mean ± SEM of 3 experiments (separate liver preparations), except for FCCP, where n=7. * Significantly different from control, $P < 0.05$, Student's t-test.

Table 2.3 **A comparison of data from these studies with those of other studies in isolated rat liver mitochondria**

Drug(s) used	Concentrations used (mM)	Maximum uncoupling concentration (mM)	% Maximum mitochondrial respiration	Reference
Indomethacin	0-2.5	0.25	194	This study
Aspirin	0-25	2.00	294	
Naproxen	0-25	3.75	149	
Ketoprofen	0-2.8	0.35	220	
Piroxicam	0-2.0 Prot=1.4-4.2mg	0.25	250	
Aspirin	0-0.5 Prot=4mg	0.5	163	Tomoda *et al.*, 1992
Aspirin	0.6 Prot=15mg	0.6	129	Mehlman *et al.*, 1972
Indomethacin	0.2 Prot=4.5mg	0.2	>300	Whitehouse and Famaey, 1973
Indomethacin	0.2 Prot=4.5mg	0.2	320	Famaey and Mockel, 1973
Indomethacin	0-1.25 Prot=7-10mg	0.25	145	Byczkowski and Korolkiewicz, 1977
Indomethacin	0.1	0.1	132	Tokumitsu *et al.*, 1977
Aspirin	1 Prot=0.6-0.65mg	1	273	
Aspirin	0-900nmoles/mg protein	900nmoles/mg protein	125	Leblondel and Allain, 1979
Indomethacin	0.05-0.3	0.1	154	Baños and Reyes, 1989
Naproxen	0.05-0.3 Prot=1-5mg	0.05-0.1	140	
Piroxicam	0-1.2 Prot=0.37-1.73 mg/ml	0.8	250	Salguiero-Pagadigorria *et al.*, 1996a

From figure 2.8 it is also apparent that the control drug paracetamol failed to stimulate oxygen consumption and only inhibited with increasing concentrations.

Study 2: The effect of the proNSAIDs fenbufen, sulindac and nabumetone, and nabumetone's metabolite 6-MNA, on mitochondrial respiration

Figure 2.9a shows the effects of fenbufen and sulindac on mitochondrial oxygen uptake. Both fenbufen and sulindac exhibited uncoupling, as shown by a stimulation in mitochondrial oxygen uptake followed by inhibition. Maximum stimulation of 165% by 1mM fenbufen and 190% by 4mM sulindac.

From figure 2.9b, it can be seen that nabumetone failed to stimulate mitochondrial oxygen consumption with concentrations reaching up to 2mM. However, the metabolite of nabumetone, 6-MNA, did uncouple in a similar manner to the conventional acidic NSAIDs, observed as a stimulation of oxygen uptake to a maximum of 180% by 0.5 mM, followed by a decline in oxygen uptake. Although this latter drop in oxygen uptake appears to be more profound than those seen with the other acidic NSAIDs tested, this is not relevant to these studies. Indeed, a less drastic inhibition of respiration may have been seen had a concentration of 6-MNA between 0.5 mM and 0.8 mM been used.

Study 3: Flurbiprofen vs NO-flurbiprofen

Flurbiprofen exhibited the characteristic stimulation in oxygen uptake, reaching a maximum of 200% of control with 2.5mM, followed by a fall in respiration. However, its NO derivative did not have such an effect over the concentration range 0 to 12mM (figure 2.10).

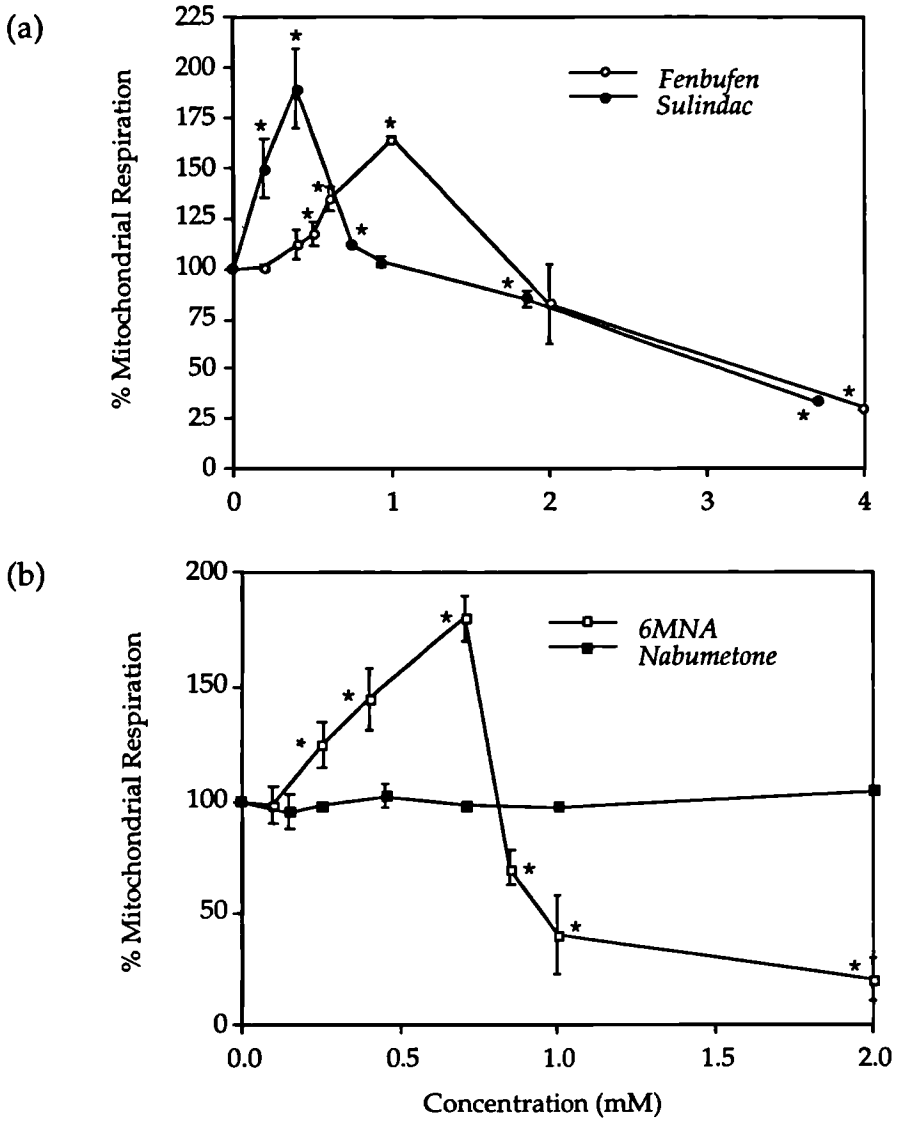


Figure 2.9 Effect of prodrugs on mitochondrial respiration

To a mitochondrial suspension (protein concentration 1.6 to 6.5 mg) was added 1mM succinate then increasing concentrations of the compounds (a) sulindac and fenbufen, and (b) nabumetone plus its metabolite 6MNA. Mitochondrial respiration was monitored at 30°C using an oxygen electrode. Each point represents the mean ± SEM of 3 experiments (separate liver preparations). * Significantly different from control, $P < 0.05$, Student's t-test.

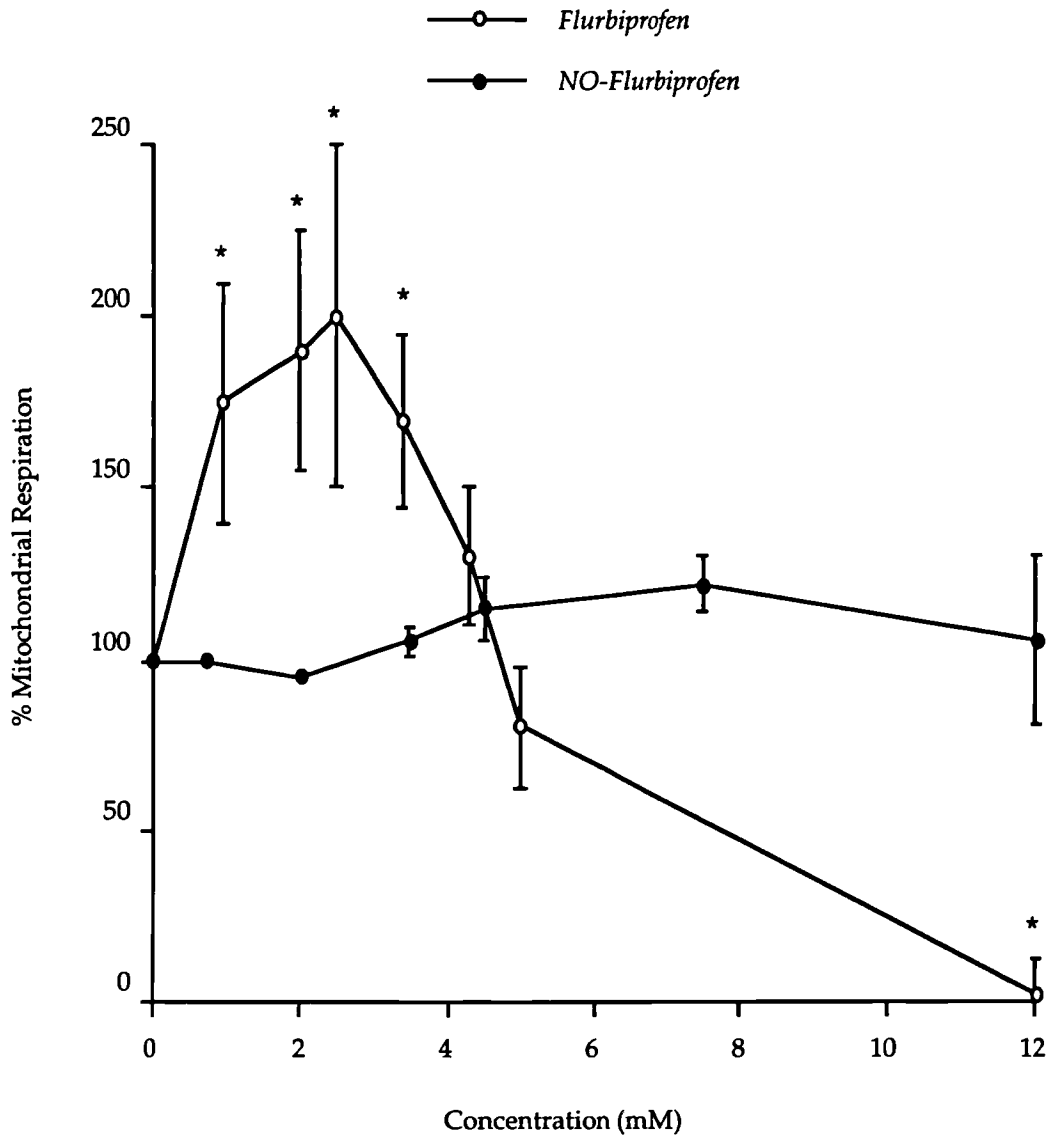


Figure 2.10 Effect of flurbiprofen and NO-flurbiprofen on mitochondrial respiration

To a mitochondrial suspension (protein concentration 0.5 to 1 mg) was added 1mM succinate then increasing concentrations of flurbiprofen or nitroxy-flurbiprofen. Using an oxygen electrode mitochondrial respiration was recorded at 30°C . Data represents the mean ± SEM of 3 experiments (separate liver preparations). * Significantly different from control, $P < 0.05$, Student's t-test.

Study 4: Effect of misoprostol on mitochondrial oxygen uptake & ultrastructure

In the experiments carried out on isolated mitochondria, the uncoupling (i.e. stimulation followed by inhibition of oxygen uptake) by indomethacin, was not prevented by misoprostol (figure 2.11a). Misoprostol itself had no effect on mitochondrial oxygen uptake. Basal respiration rate (100%) was retained. It must be noted, however, that despite these experiments being performed on 3 separate liver preparations as before, the data had relatively large error bars. The reason for this is uncertain, but may have been decreased had more experiments been done.

Figure 2.11b shows how the mitochondria were viewed under EM. It was found that in the control rats and rats treated with misoprostol alone, less than 5% of the enterocytes had abnormal mitochondria. Misoprostol itself had no significant effect on intestinal mitochondrial morphology (figure 2.11bi). (Control mitochondria are not shown in this figure as the EM images were identical to 2.11bi). In those rats treated with indomethacin alone 60-70% of the cells contained abnormal mitochondria (EM image not shown). This was not significantly different when indomethacin was coadministered with misoprostol which had 50-70% of the cells containing damaged mitochondria. As shown by severity of the damage seen in figure 2.11bii, addition of misoprostol did not attenuate the mitochondrial structural changes caused by indomethacin.

Study 5: Effect of glucose-citrate on mitochondrial oxygen uptake & ultrastructure

In isolated mitochondria, the pattern of stimulation then inhibition of respiration by indomethacin was similar when added in the presence or absence glucose and citrate (figure 2.12a).

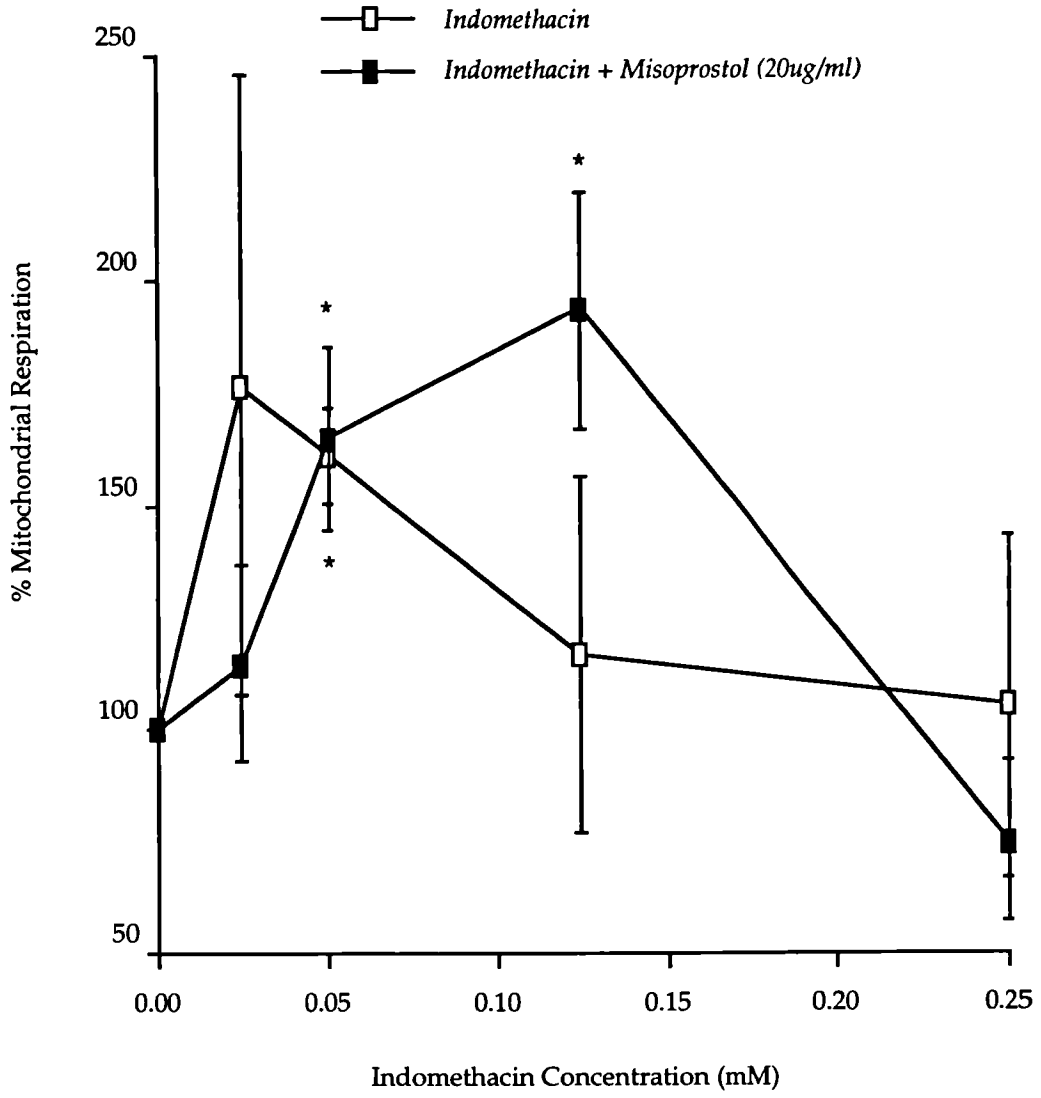
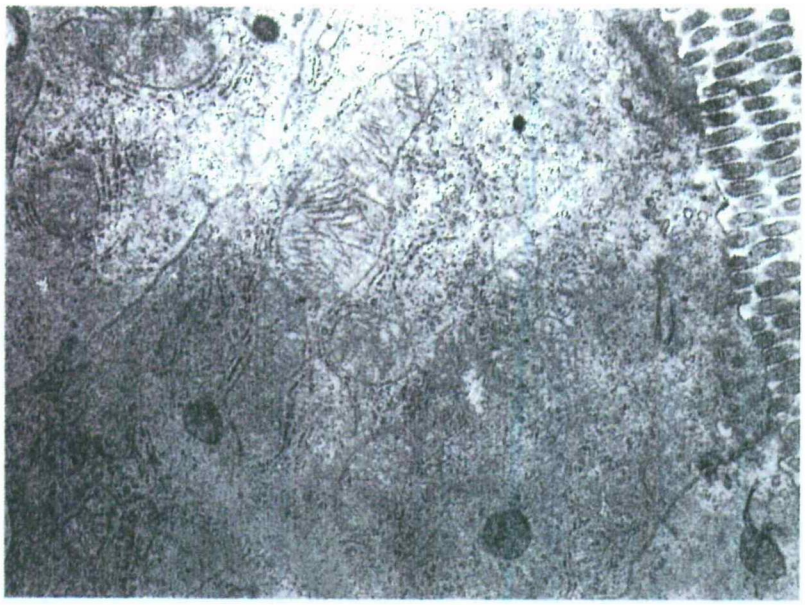


Figure 2.11a Effect of misoprostol on indomethacin-induced uncoupling of oxidative phosphorylation

To mitochondria (1.2 to 1.7 mg) was added 1mM succinate then increasing concentrations of either indomethacin or indomethacin mixed with 20µg/ml misoprostol. Mitochondrial respiration was monitored using an oxygen electrode at 30°C. Results are mean ± SEM of 3 experiments (separate liver preparations). * Significantly different from control, $P < 0.05$, Student's t-test.

(i)



(ii)

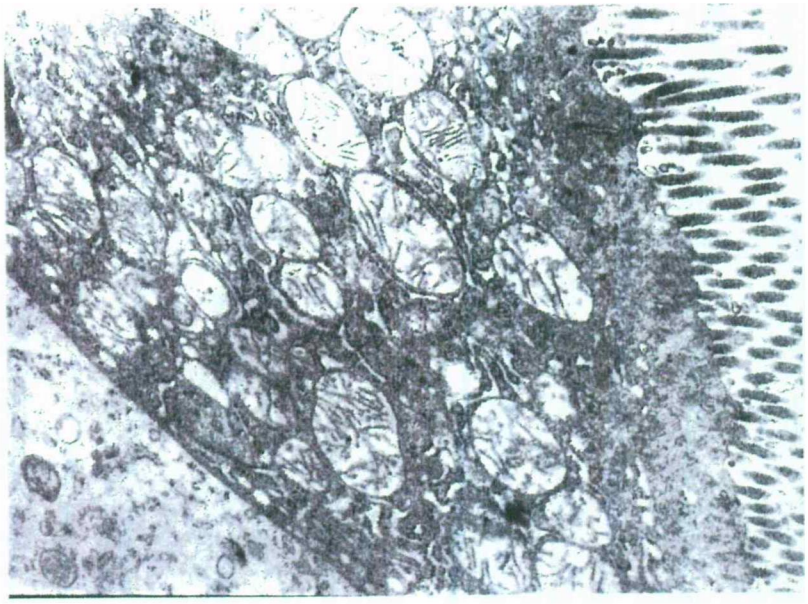


Figure 2.11b EM images showing effect of misoprostol and indomethacin on mitochondria

Rats were orally administered (i) 60 µg/kg body weight misoprostol or (ii) 10 mg/kg body weight indomethacin plus misoprostol. EM was then performed to visualise intestinal mitochondria. (See main text for experimental details). Magnification x43,000. Source: EM unit, Northwick Park Hospital, Middlesex.

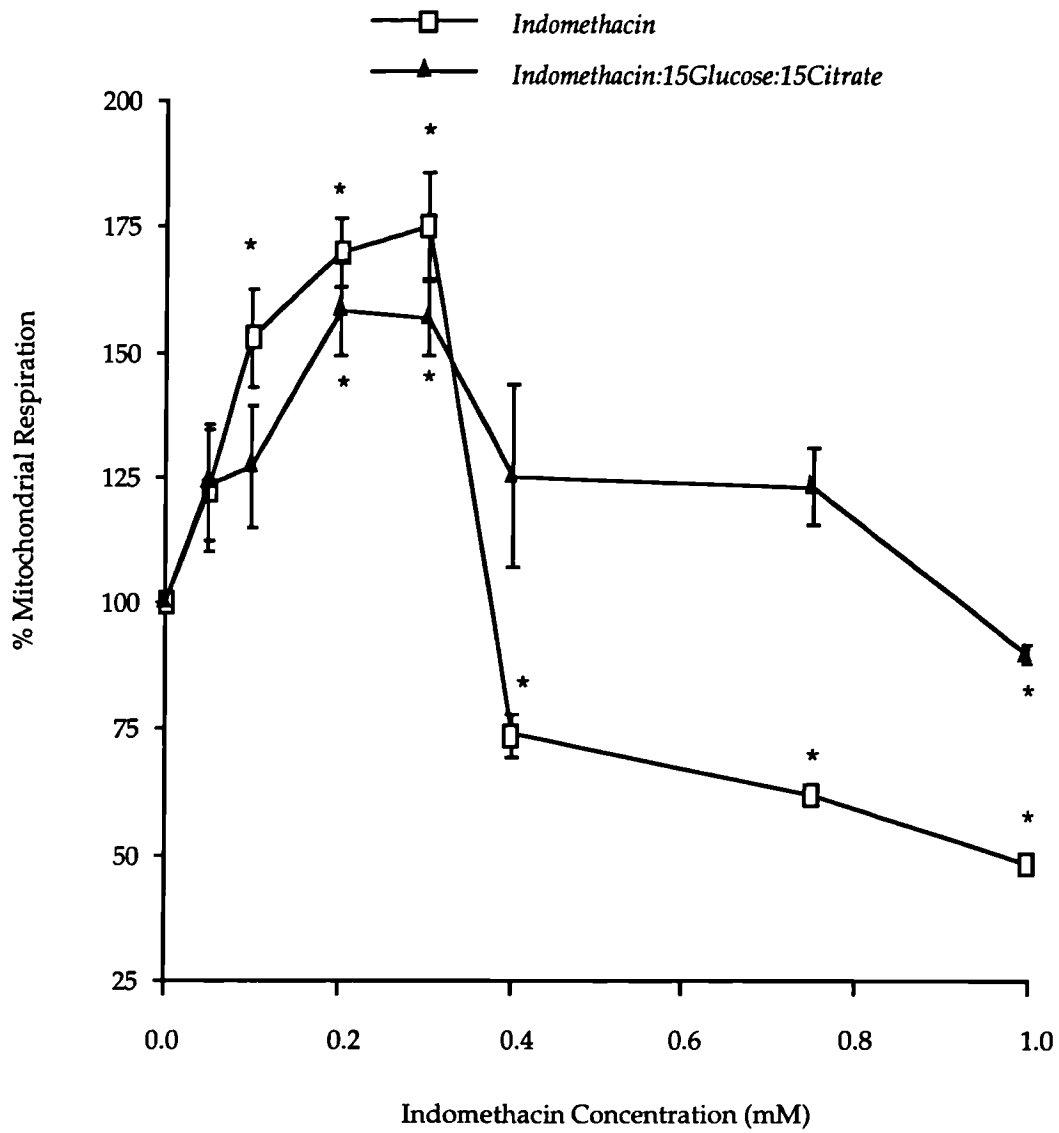


Figure 2.12a The effect of glucose and citrate on indomethacin-induced uncoupling of oxidative phosphorylation

To mitochondria (1.1 to 1.5 mg) was added 1mM succinate then increasing concentrations of either indomethacin or indomethacin mixed with 15 times its weight of glucose and citrate. Mitochondrial respiration was monitored at 30°C using an oxygen electrode. Each point represents the mean ± SEM of 3 experiments (separate liver preparations). * Significantly different from control, $P < 0.05$, Student's t-test.

Under EM, the amount of enterocytes containing abnormal mitochondria was less than 5% in the control and glucose/citrate-treated rats (images not shown). In the indomethacin-treated rats, 50-70% of the enterocytes had abnormal mitochondria, exhibiting severe mitochondrial damage i.e. ballooning, distension and vacuolisation (figure 2.12bi). When indomethacin was coadministered with glucose and citrate, 65% of the enterocytes had abnormal mitochondria, although there was no significant attenuation of the mitochondrial damage induced by indomethacin alone (figure 2.12bii).

2.7 Discussion

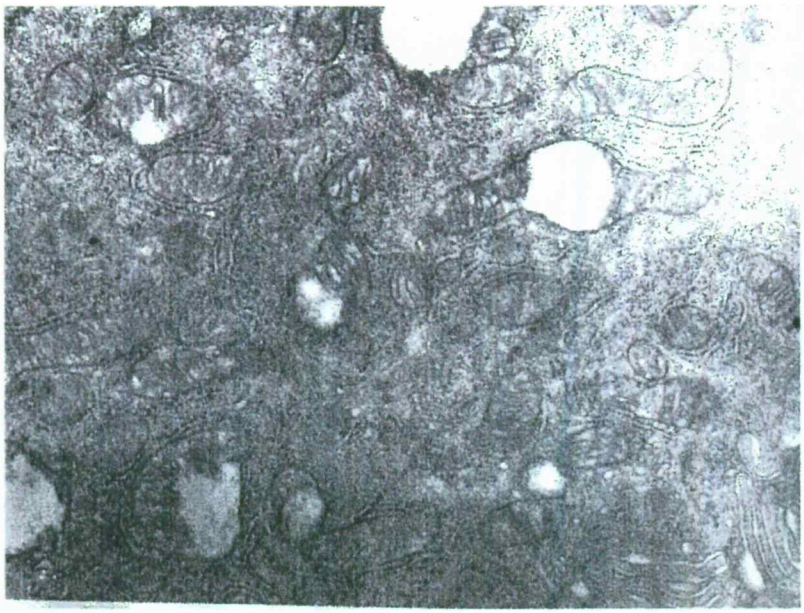
Study1

All the conventional acidic NSAIDs studied uncoupled oxidative phosphorylation. As with most 'inhibitory uncouplers', in isolated mitochondria, this was observed as a characteristic biphasic response - an initial stimulation followed by an inhibition of oxygen consumption. The rise in oxygen uptake corresponded to the uncoupling effect of the drug, whereas the fall related to an inhibition of electron transport.

Table 2.3 compares the data from this study with those of earlier work. In the main, the concentrations required to give maximal stimulation in oxygen uptake found by the present study, are very similar to those found by other groups. In addition, concentrations of acidic NSAIDs have been measured *in vivo* in the rat gastric mucosa and found to be approximately in the same range as their uncoupling concentrations (Frey and El-Sayed, 1977).

Some groups have also reported that aspirin does not uncouple isolated rat liver mitochondria (Thompkins and Lee, 1969; Spenney and Bhown, 1977;

(i)



(ii)

Figure 2.12b EM images showing effect of glucose/citrate and indomethacin on mitochondria

Rats were orally given (i) 30 mg/kg body weight indomethacin or (ii) 450 mg/kg body weight glucose/citrate and 30 mg/kg body weight indomethacin. Enterocyte mitochondria were then viewed by EM. (See main text for experimental details). Magnification x43,000. Source: EM unit, Northwick Park Hospital, Middlesex.

and Keller *et al.*, 1992). However, in the study by Spenney and Bhown (1977), there was a slight stimulation of mitochondrial respiration with 5.6 mM aspirin, albeit insignificantly (P = 0.10). In addition, Thompkins and Lee (1969), showed up to a 30% reduction in P/O ratio with 3 mM aspirin, again suggestive of an uncoupling of oxidative phosphorylation.

Paracetamol was used as the control in the present experiments since, though as effective as many of the NSAIDs as an analgesic and antipyretic agent, it lacks most of the side-effects of the NSAIDs and is non-acidic. The analgesic/antipyretic properties of paracetamol may be due to its inhibition of prostaglandin production in the central nervous system. However, paracetamol is not antiinflammatory (Clissold, 1986), probably because it does not inhibit peripheral prostaglandin synthesis (Marshall *et al.*, 1987).

The present experiments show that NSAIDs which are known to cause gastrointestinal damage also uncouple oxidative phosphorylation, whereas paracetamol neither causes GI lesions nor does it uncouple. Paracetamol also failed to uncouple and induce swelling in rat kidney mitochondria (Mingatto *et al.*, 1996; and Uyemura *et al.*, 1997). Therefore, this would suggest a causal connection between uncoupling and GI damage (see also study 2 below). Other authors, however, have reported that paracetamol can impair mitochondrial oxidative phosphorylation (Meyers *et al.*, 1988; and Katyare and Satav, 1989).

Paracetamol does inhibit coupled mitochondrial respiration in these studies. Similarly, 10 mM paracetamol has been shown to inhibit respiration by 60% (Ramsay *et al.*, 1989). In addition, in mouse liver mitochondria, 5 mM paracetamol caused significant inhibition of respiration and this occurred prior to the loss of cell membrane integrity (Burcham and Harman, 1991). In

kidney mitochondria, paracetamol gave a small (95% of control) but consistent inhibition respiration (Porter and Dawson, 1979). In all of the above, the inhibition of state 4 respiration was similar in glutamate/malate- or succinate-supported respiration. However, the biological significance of this inhibition by paracetamol is uncertain as in all cases it only occurred to a significant extent at relatively high concentrations.

Study 2

The suggestion that prodrugs produce less gastrointestinal side-effects than conventional NSAIDs, was thought to be because the gastric or intestinal mucosa is not exposed to high local concentrations of the active antiinflammatory compound during drug absorption. However, a small percentage of patients treated with the prodrugs (20% with sulindac and 12% with fenbufen) do still have GI complaints (Brogden *et al.*, 1981; and Insel, 1996). In fact, sulindac is only better tolerated than the other NSAIDs in short-term endoscopy studies (Lanza, 1989): in the long-term it produces ulcers and other complications (Larkai, 1987).

Thus, in theory, prodrugs may not reduce the "topical" damage if it is caused by a separate mechanism (Insel, 1996), such as uncoupling of oxidative phosphorylation (see section 1.6.1.1, chapter 1). This is substantiated by the present study which showed that the prodrugs fenbufen and sulindac uncoupled mitochondrial oxidative phosphorylation.

6MNA was also found to stimulate coupled respiration, whereas its parent compound nabumetone did not. 6MNA, fenbufen and sulindac all possess a carboxyl group unlike nabumetone. As previously mentioned the free acidic group (generally the carboxylic acid group) of NSAIDs is required for uncoupling activity. The acidic side chain appears not only to contribute to

uncoupling but may also play an important role in the formation of gastrointestinal lesions. This has been supported by the fact that altering the acidic moiety of NSAIDs by esterification, appears to reduce the gastric toxicity of these drugs without affecting their antiinflammatory activity (Whitehouse and Rainsford, 1980). Nabumetone does not produce the GI damage seen with other acidic NSAIDs in rats (Boyle *et al.*, 1982) and some studies in humans also suggest greater gastrointestinal tolerability (Melarange *et al.*, 1992).

Study 3

This study was carried out not only to validate earlier *in vivo* findings, but also to see the direct effect that modification of the NSAID carboxylic acid group would have on uncoupling. As expected, flurbiprofen possessing an acid side chain, uncoupled oxidative phosphorylation in isolated mitochondria. NO-flurbiprofen did not uncouple possibly due to addition of the NO group rendering the NSAID non-acidic and hence ineffective as a protonophore (uncoupler).

However, in another study, electron microscopic examination revealed a similar pattern of damage in mitochondria treated *in vivo* with either flurbiprofen or NO-flurbiprofen (Somasundaram *et al.*, 1997). In addition, the two compounds also increased intestinal permeability to the same extent. The apparent discrepancy between these reports and the present *in vitro* findings could be reasonably explained as due to the abundant presence of intestinal esterases. These enzymes (absent in isolated mitochondria) can cleave the ester bond in the NO compound yielding flurbiprofen, which would then be free to exert its detrimental biochemical effects during drug absorption (Somasundaram *et al.*, 1997). The concomitant release of the NO group will then help to prevent the reduction in mucosal blood flow and neutrophil recruitment at a later stage of NSAID-induced gastrointestinal injury (figure

2.13). It therefore appears that it is not only the release of nitric oxide from NO-flurbiprofen which is important for its improved gastrointestinal tolerability over flurbiprofen, but also the modification of the carboxylic acid side chain.

Furthermore, in the present study, NO itself (added as a saturated aqueous solution at 1mM stock) was found not to uncouple mitochondria (data not shown). Interestingly, however, low (physiological) concentrations of NO reversibly suppresses mitochondrial respiration probably by binding to cytochrome a_3, producing a transient inhibition of electron flow (Cassina and Radi, 1995). Then higher (cytotoxic) concentrations of NO irreversibly inhibit respiration at complexes I, II and III. The latter effect is thought to be mediated by peroxynitrite, the product of a reaction of mitochondrial superoxide with NO. This ability to inhibit mitochondrial respiration could explain some of the beneficial roles of NO; e.g. NO released under inflammatory conditions may be useful for host defense by being able to suppress bacterial respiration (Xie *et al.*, 1996).

Study 4

It is suggested that uncoupling of mitochondrial oxidative phosphorylation is involved in the "topical" damage seen with NSAIDs (Somasundaram *et al.*, 1997). The present study demonstrates that misoprostol does not prevent NSAID-induced uncoupling *in vitro* or *in vivo*, suggesting that uncoupling is a prostaglandin-independent process. Indeed, prostaglandins do not prevent the immediate widespread "topical" destruction of the surface epithelium caused by irritants such as ethanol (Lacy, 1982). Instead, misoprostol protects by retaining the integrity of the remaining layers of the mucosa. That is, the compound enhances mucosal defense mechanisms and inhibits acid

secretion, thereby preventing deeper necrotic and haemorrhagic lesions (Konturek and Konturek, 1994).

Misoprostol has also been found to be ineffective at preventing ethanol-induced inhibition of state 3 respiration in isolated rat liver mitochondria respiring on succinate or ascorbate (Dlugosz *et al.*, 1991). However, with glutamate and malate as the substrates, there was partial protection with misoprostol, probably because ethanol-induced inhibition is most profound at complex I (Cederbaum *et al.*, 1974).

The present experiments also show that misoprostol alone does not uncouple oxidative phosphorylation. Nevertheless, prostaglandin E_1 given to rats does appear to cause a reduction in the total number of mitochondria as well as an increase in irregularly-shaped mitochondria (Ozeki *et al.*, 1987). One reason for this discrepancy may be that naturally-occurring prostaglandins are acidic whereas the synthetic derivative misoprostol is not, though this may be an over-simplified explanation. This is because in another study, in accordance with the present study, it was reported that the prostaglandins D_2, E_2, $F_{2\alpha}$ and E_1, had no effect on the respiratory activity of isolated rat brain mitochondria (Takeuchi *et al.*, 1991).

Study 5

It has been suggested that the NSAID-induced increase in intestinal permeability will expose the mucosa to luminal substances with subsequent inflammation (Somasundaram *et al.*, 1995). Glucose and citrate coadministered to human volunteers with indomethacin in a ratio of 15:15:1, has been shown to reduce the permeability changes seen with indomethacin alone (Bjarnason *et al.*, 1992). In addition, indorise, a combination of indomethacin, glucose and citrate, given to rats, completely prevents

permeability changes and ulcer formation induced by indomethacin (Hayllar *et al.*, 1991).

The underlying biochemical mechanism for this protection is unclear. One possibility is that salicylate inhibits some of the steps in the TCA cycle (Kaplan *et al.*, 1954). It is possible, therefore, that salicylates and other NSAIDs may competitively inhibit enzymes in glycolysis and the tricarboxylic acid (TCA) cycle, causing a reduction in ATP production and hence cell death (Rainsford and Whitehouse, 1980). The presence of glucose and citrate in the intestinal lumen would provide the enterocytes with substrates for glycolysis and the TCA cycle, thereby overcoming the competitive effects of the NSAIDs. However, the structural diversity of NSAIDs, make it unlikely though not impossible. NSAIDs certainly competitively inhibit cyclooxygenase despite their chemical diversity, although this may be due to the interaction between the carboxyl group on the NSAID with the NH_3-arginine group on cyclooxygenase (Picot *et al.*, 1994).

An alternative explanation is that citrate, being a potent inhibitor of phosphofructokinase, a rate-controlling enzyme of glycolysis, may divert glucose metabolism along the hexose monophosphate pathway (see figure 4.6, chapter 4). This would provide reducing power to prevent NSAID-induced oxygen radical damage, a mitochondrial-independent process (Bjarnason *et al.*, 1992). In this case, glucose and citrate might protect against some NSAID damage, but not by any effect on NSAID uncoupling of oxidative phosphorylation.

A controlling factor of the activity of the TCA cycle is the NADH/NAD ratio. Thus when uncouplers, including NSAIDs, accelerate mitochondrial respiration, the decrease in NADH/NAD ratio would produce a

compensatory stimulation of the TCA cycle (Fromenty *et al.*, 1993). In addition, uncoupling mitochondria would lower ATP levels and stimulate glycolysis to produce the necessary ATP for the cell. Numerous NSAIDs have been found to stimulate glycolysis (Brass and Garrity, 1985; Kemmelmeier and Bracht, 1989; Salguiero-Pagadigorria *et al.*, 1996a; and Petrescu and Tarba, 1997). This is in sharp contrast to the earlier suggestion that NSAIDs inhibit the TCA cycle and glycolysis. Addition of glucose and citrate in the present experiments did not affect the uncoupling induced by indomethacin suggesting, as above, that the protective effect of glucose and citrate is not a direct protection at the mitochondrial level but an effect related to whole cell metabolism. However, these arguments do not explain why glucose and citrate needed to given together in order to have a protective effect.

Cooney and Dawson (1977) found that when D-glucose was added as the sole exogenous substrate, 2,4-DNP stimulated oxygen uptake, consistent with its uncoupling activity. However, indomethacin, at concentrations previously shown to uncouple oxidative phosphorylation in isolated mitochondria (Whitehouse, 1964b; Famaey and Mockel, 1973; and Byczkowski and Korolkiewicz, 1977), inhibited oxygen consumption. Cooney and Dawson (1977) went on to demonstrate that this inhibition of oxygen uptake was not caused by direct inhibitory effects of indomethacin on the TCA cycle (as previously mentioned) but by the inhibition of the transfer of reducing equivalents (e.g. NADH) from the cytoplasm to the mitochondria. They showed that the effect of indomethacin on glucose metabolism was independent of its ability to uncouple oxidative phosphorylation. This again is consistent with the findings of the present studies, since glucose and citrate failed to have any effect on the uncoupling induced by indomethacin.

Finally, with the intestinal enterocyte, it is possible that not all the mitochondria will be uncoupled by NSAIDs. Any remaining intact mitochondria will still be able to utilise glucose and citrate for glycolysis and TCA cycle activity, thereby alleviating the deleterious effects of NSAIDs.

Justifying the Use of Rat Liver Mitochondria

As the purpose of this study was to investigate one of the underlying mechanisms of NSAID-induced gastrointestinal lesion, the use of mitochondria isolated from rat liver may be questioned. However, it has been shown that mitochondria isolated from various sources and species have generally similar properties with respect to electron transport and oxidative phosphorylation (Brody, 1955). For example, NSAIDs also uncouple oxidative phosphorylation of rat brain and kidney mitochondria (Brody, 1956; and Mingatto *et al.*, 1996). The extent to which the NSAIDs diflunisal, flufenamic acid and mefenamic acid stimulate respiration in liver mitochondria isolated from rat, mouse, rabbit and guinea-pig is similar in all four species (McDougall *et al*, 1983). In addition, aspirin uncouples mitochondria isolated from the gastric mucosa of pigs at a similar concentration range to the studies of this thesis (Jörgensen *et al.*, 1976). Aspirin has even been found to cause an increase in oxygen consumption in whole tissue, such as rat liver slices, at the lower concentrations of 1.49mM and 2.97mM, and a progressive decrease in oxygen uptake at the higher concentrations of 5.94mM and 7.45mM (Fishgold *et al.*, 1951). These experimental findings closely parallel those of the present study using aspirin and isolated rat liver mitochondria.

Several attempts to prepare mitochondria from gut (jejunal) tissue were made but were unsuccessful with regard to coupling ability. Problems of preparation due to the action of endogenous proteases and the high concentrations of mucus in intestinal preparations have been previously

noted (Sherratt, 1963). One advantage of using kidney mitochondria would be the high content of epithelial tissue. However the majority of previous work on NSAIDs and mitochondria had used liver mitochondria and/or liver slices (see tables 2.3 and 3.1). It was therefore decided, for the present work, to use liver mitochondria to aid direct comparison.

CHAPTER THREE

INHIBITION OF MITOCHONDRIAL ELECTRON TRANSPORT BY NSAIDS

The *in vitro* studies on the effects of NSAIDs on mitochondrial function (chapter 2), suggest that these compounds may uncouple oxidative phosphorylation and/or inhibit the respiratory chain. Either action would, in theory, cause changes to mitochondrial ultrastructure *in vivo*, and a decrease in mucosal ATP which, in turn, would lead to an increase in intestinal permeability. Such effects are key steps in the proposed pathogenesis of NSAID-induced damage to the gastrointestinal tract. This chapter looks specifically at the action of NSAIDs on the respiratory chain.

Electron transport and oxidative phosphorylation can be envisaged as two separate processes, required to be tightly coupled for the purpose of ATP generation. Hence, in order to investigate selectively the effects of NSAIDs on the electron transport, the mitochondria were first uncoupled with FCCP so that no oxidative phosphorylation could take place. To localise the possible site(s) of inhibition along the respiratory chain by the drugs, specific respiratory chain substrates and inhibitors were used. Inhibitors, unlike uncouplers of oxidative phosphorylation, act on a specific component of the respiratory chain (Spenney and Bhown, 1977).

3.1 Exogenous Respiratory Chain Substrates

3.1.1 Glutamate and Malate

NADH produced by glycolysis, cannot readily cross the mitochondrial membrane (Mathews and van Holde, 1996). In order for NADH to donate electrons to the respiratory chain it must somehow be able to transfer reducing equivalents into the mitochondrion for reoxidation. This process occurs through the "malate-aspartate shuttle" and is particularly active in the

heart and liver (figure 3.1). In short, in this shuttle, NADH reduces oxaloacetate to malate in the cytoplasm. Malate then enters the mitochondria, where it is reoxidised, regenerating NADH and oxaloacetate. Because oxaloacetate cannot cross the mitochondrial membrane to pass out of the mitochondria, it is transaminated to aspartate, which is then transported out into the cytoplasm for reconversion to oxaloacetate, beginning the cycle again. The involvement of transamination in this shuttle means that α-ketoglutarate must be continuously transported out of mitochondria and glutamate continuously transported in.

The presence of glutamate is also important for this shuttle to proceed since, like malate, it too can pass across the mitochondrial membrane into the mitochondria and be transaminated to aspartate. Therefore, in isolated mitochondria, as endogenous substrates are used up, glutamate and malate are provided in equal amounts to generate NADH within the matrix, thereby reducing complex I of the electron transport chain. Electrons are then transferred from complex I to complex III and eventually to complex IV, resulting in the reduction of molecular oxygen to water (see figure 2.2, chapter 2).

3.1.2 Succinate

Addition of succinate elicits FAD-linked oxidation. Therefore, electrons are donated to complex II, from where they are passed onto complex III and then complex IV (see figure 2.2, chapter 2).

3.1.3 Ascorbate and TMPD

N:N:N':N' tetramethyl p-phenylenediamine (TMPD) is a redox carrier that

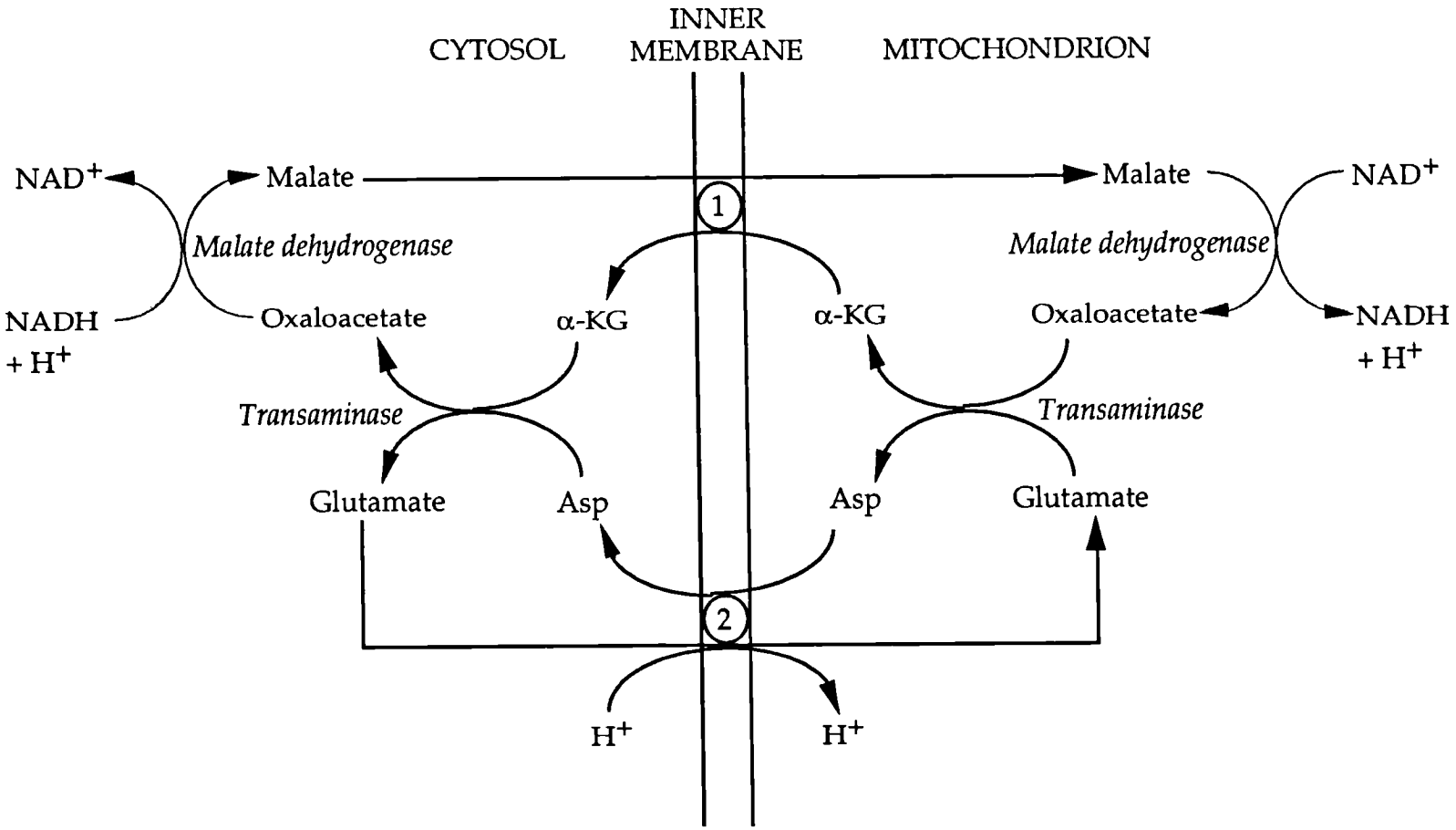


Figure 3.1 The malate/aspartate shuttle
This shuttle system enables the transfer of reducing equivalents from the cytosol into the mitochondrion. *(1) = Ketoglutarate transporter; (2) = glutamate-aspartate transporter; α-KG = α-ketoglutarate; and Asp = aspartate.* Redrawn from Mayes (1993).

transfers electrons directly to cytochrome *c* bound to cytochrome oxidase (complex IV). But only when TMPD is given together with ascorbate is cytochrome oxidase activity fully saturated since ascorbate maintains TMPD in the reduced state (Kimelberg and Nicholls, 1969) (figure 3.2). However, TMPD, a purple-coloured free radical, has itself also been found to uncouple oxidative phosphorylation at a concentration of 0.2 mM (Park *et al.*, 1957).

3.2 Exogenous Respiratory Chain Inhibitors

3.2.1 Rotenone

Rotenone is a powerful insecticide and fish poison, extracted from the roots of the *Derris* plant found in South America (Mayes, 1993). The compound is a specific, non-competitive and high affinity inhibitor of complex I, blocking electron flow from the iron-sulphur centres of complex I to coenzyme Q (ubiquinone, UQ), possibly by acting as UQ analogues. The inhibitor is believed to bind near the site of ubiquinone reduction in the distal portion of complex I (Greenamyre *et al.*, 1992). A concentration of 30 nmoles/gram protein of rotenone is sufficient to inhibit respiration in rat liver mitochondria and higher concentrations do not uncouple succinate or ascorbate/TMPD-supported respiration. Rotenone, being hydrophobic, is normally dissolved in ethanol for use (Slater, 1967).

3.2.2 Antimycin A

Antimycin A is a family of fungicidal antibiotics produced by a species of *Streptomyces* , comprising of a mixture of at least four closely related antibiotics known as antimycin A_1, A_2, A_3 and A_4 (Tyler, 1992). Antimycin A

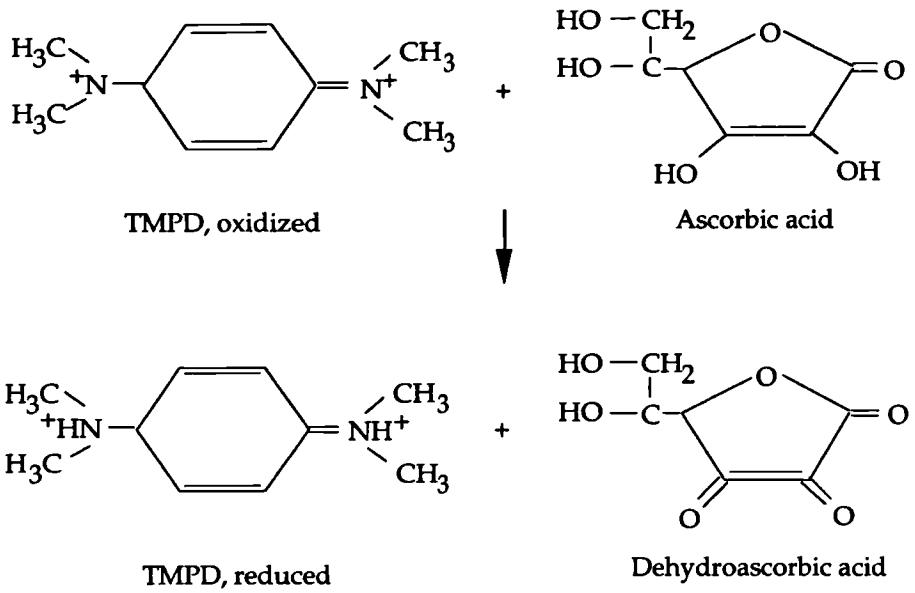


Figure 3.2 Ascorbate and TMPD

Ascorbate reduces TMPD which can then transfer electrons directly to cytochrome *c* (complex IV) of the respiratory chain. From Voet and Voet (1995).

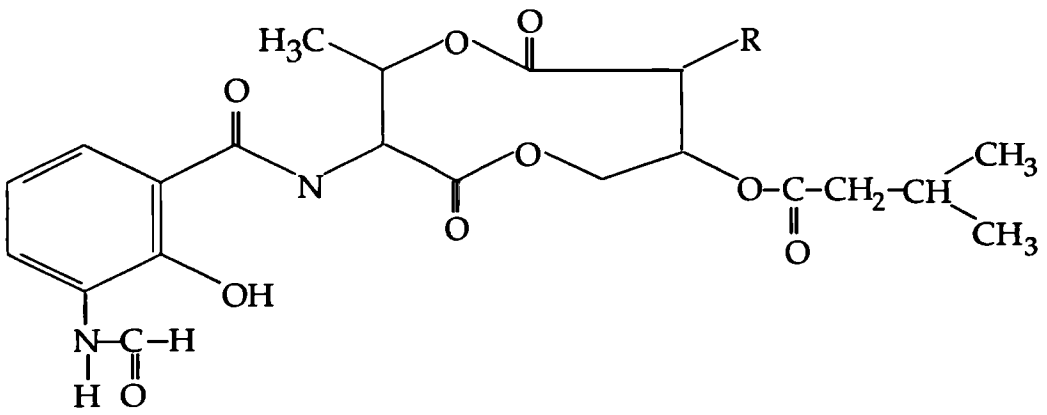


Figure 3.3 Structure of antimycin A

Antimycin A blocks electron transport at complex III of the respiratory chain. In antimycin A_1: R = *n*-hexyl; antimycin A_2: R = *n*-butyl.

is a potent inhibitor which specifically inhibits the respiratory chain between cytochrome *b* and cytochrome c_1 of complex III (figure 3.3; and Slater, 1973). Maximal inhibition by antimycin A occurs after 1 minute (Slater, 1967). The literature also suggests that antimycin A be dissolved in ethanol and that no more than 0.2 µmole be used per gram of mitochondrial protein be used in inhibition studies, since higher concentrations of the compound will uncouple ascorbate/TMPD-supported respiration.

3.2.3 Cyanide

The classic poison cyanide is a powerful reversible inhibitor of oxidised cytochrome oxidase (complex IV), totally arresting respiration (Mayes, 1993). Cyanide reacts specifically with the sixth coordinate of the haem *a* iron of cytochrome a_3, the terminal electron carrier of the respiratory chain.

3.3 Aims of the Studies

Inhibition of mitochondrial respiration by NSAIDs was investigated using FCCP-uncoupled mitochondria. In addition, an attempt was also made to pinpoint the site along the respiratory chain where the NSAIDs may be binding. In order to achieve this three different techniques were employed: the oxygen electrode, the ferricyanide reductase system and electron paramagnetic resonance (EPR).

3.4 Oxygen Electrode Studies

3.4.1 Materials and Methods

Materials and methods are essentially the same as in section 2.4 (chapter 2), with the addition of :

Reagents

The substrates sodium (Na) glutamate, Na malate; inhibitors rotenone and antimycin A were purchased from the Sigma Chemical Company Ltd., Dorset, U.K. The inhibitor Na cyanide was supplied by the Aldrich Chemical Company, Inc., Wisconsin, U.S.A.

Solubulisation of the Compounds

The substrates glutamate and malate, and the inhibitor cyanide, were prepared as 1M stock solutions in distilled water. The inhibitor rotenone was prepared as a 10mM stock solution and antimycin A as a 1mg/ml stock solution, by dissolving in 100% ethanol. To see effective inhibition, higher concentrations of stock solutions for indomethacin and aspirin were used, 250mM and 500mM respectively (prepared as before).

Experimental Procedure

Isolation of rat liver mitochondria, oxygen electrode calibration and P/O and RCR measurement were carried out as before. The following studies were then performed:

Study 1 - Effects of mitochondrial substrates and inhibitors

This was carried out to establish the order of electron flow through the complexes of the respiratory chain and the positions blocked by various site-specific inhibitors. This also served to test that the electron transport chain of this mitochondrial preparation was operating adequately.

To 1ml suspension mixture was added 100µl isolated mitochondria (protein concentration 5.2mg) then the following sequence of substrates and inhibitors to give the final concentrations:

(i) 10mM glutamate plus 10mM malate (complex I substrates),

(ii) 20µM rotenone (complex I inhibitor),

(iii) 1mM succinate (complex II substrate),

(iv) 10µM antimycin A (complex II inhibitor),

(v) 2mM ascorbate and 20µM TMPD (complex IV substrates) and

(vi) 1mM cyanide (complex IV inhibitor).

Study 2 - Effect of drugs on complex I

The effect of the drugs on electron transport from complex I to III and finally to IV was measured by using a mixture of glutamate and malate to stimulate mitochondrial respiration at complex I, and FCCP to uncouple the mitochondria. The additions were: 100µl mitochondria (2.9 to 6.7 mg), 10mM glutamate plus 10mM malate, 0.1 µM FCCP then the drugs indomethacin, aspirin, naproxen and paracetamol with final concentrations ranging from 0 to 115mM.

Study 3 - Effect of drugs on complex II

The effect of the drugs on electron transport from complex II to complex III through to complex IV was monitored using succinate as substrate. Rotenone was used to prevented any electrons from being transferred from complex I

and FCCP again ensured the mitochondria were uncoupled prior to addition of the drug. The additions were: 100µl mitochondria (2.9 to 6.7 mg), 20µM rotenone, 1mM succinate, 0.1 µM FCCP then 0 to 190mM of the drugs indomethacin, aspirin, naproxen and paracetamol.

As before, each different concentration of drug was added to a fresh sample of mitochondria and solutions added never exceeded 200µl. After every addition a period of approximately 1-2 minutes was allowed to elapse in order to observe clear rates for oxygen uptake on the chart recorder. FCCP is a specific and potent uncoupler of mitochondrial oxidative phosphorylation whose concentration was kept deliberately low, since addition of too much would itself result in inhibition of respiration (Slater, 1967).

3.4.2 Results

Study 1

Figure 3.4 shows the oxygen electrode trace of how the different substrates and inhibitors affected mitochondrial oxygen uptake. The rate of respiration given by each compound is denoted on the trace. The addition of 100 µl mitochondria (protein concentration 5.2mg), gave a relatively slow basal rate of respiration, recorded as slight reduction in oxygen uptake.

(i) Injecting a mixture of glutamate and malate into the chamber, enabled NADH generation within the mitochondria, thereby donating electrons to complex I. This caused an slight but further increase in respiration.

(ii) Addition of the complex I inhibitor rotenone completely lowered complex I-mediated oxygen uptake.

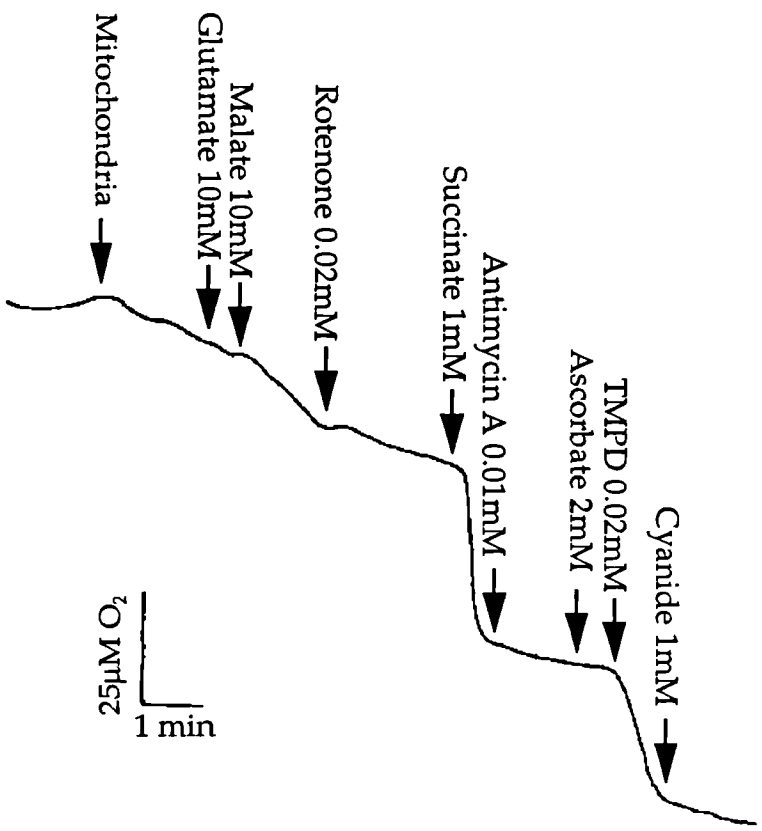


Figure 3.4 Effect of site-specific substrates and inhibitors on mitochondrial oxygen uptake

Oxygen electrode trace obtained when substrates and inhibitors are injected, to the final concentrations shown, into the sample chamber containing mitochondria. Arrows indicate the various additions.

(iii) By adding succinate supplied electrons to complex II, therefore oxygen consumption was again enhanced. The fact that electrons from $FADH_2$ are still able to reduce O_2 in the presence of rotenone indicate that electrons from $FADH_2$ enter the electron transport chain after the rotenone-blocked step (Voet and Voet, 1995).

(iv) Antimycin A inhibited the succinate-stimulated respiration as expected from its known action on cytochrome bc_1.

(v) Addition of ascorbate and TMPD to the antimycin A-inhibited mixture resulted in a resumption of oxygen consumption since ascorbate and TMPD can directly reduce complex IV.

(vi) Oxygen uptake was arrested by adding cyanide to the system, inhibiting complex IV activity.

Study 2

All the drugs inhibited mitochondrial respiration in a concentration-dependent manner when electron entry through complex I was used. Figure 3.5 shows how the inhibition was observed on the oxygen electrode trace. Only (a) indomethacin and (b) aspirin are represented since a similar picture was seen with naproxen and paracetamol (not shown). In figure 3.6 these changes have been converted to percentage of control. According to their IC_{50}'s (mM), the potency of the drugs at inhibiting electron transport, initiating from complex I, was:

indomethacin (0.3) » paracetamol (10.7) ≈ aspirin (11) › naproxen (13).

Study 3

The drugs also inhibited mitochondrial respiration with increasing concentration when electron entry was via complex II using succinate as a substrate. All the drugs again inhibited in a similar manner and, as an example, figure 3.7 shows how paracetamol-mediated inhibition was seen on

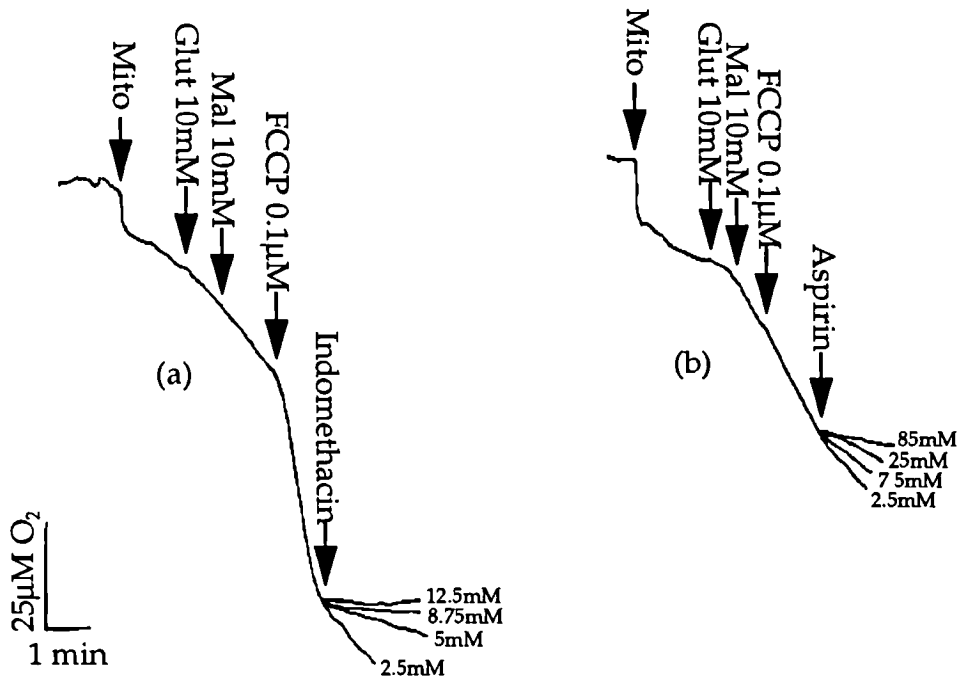


Figure 3.5 Effect of drugs on complex I-mediated mitochondrial respiration

These are oxygen electrode traces showing how (a) indomethacin and (b) aspirin affected glutamate/malate-stimulated mitochondrial oxygen uptake. Arrows indicate additions made to mitochondria (2.9 to 6.7 mg protein). FCCP was added prior to the NSAID, to uncouple the mitochondria. *Abbreviations: Mito = mitochondria, Glut = glutamate, Mal = malate.*

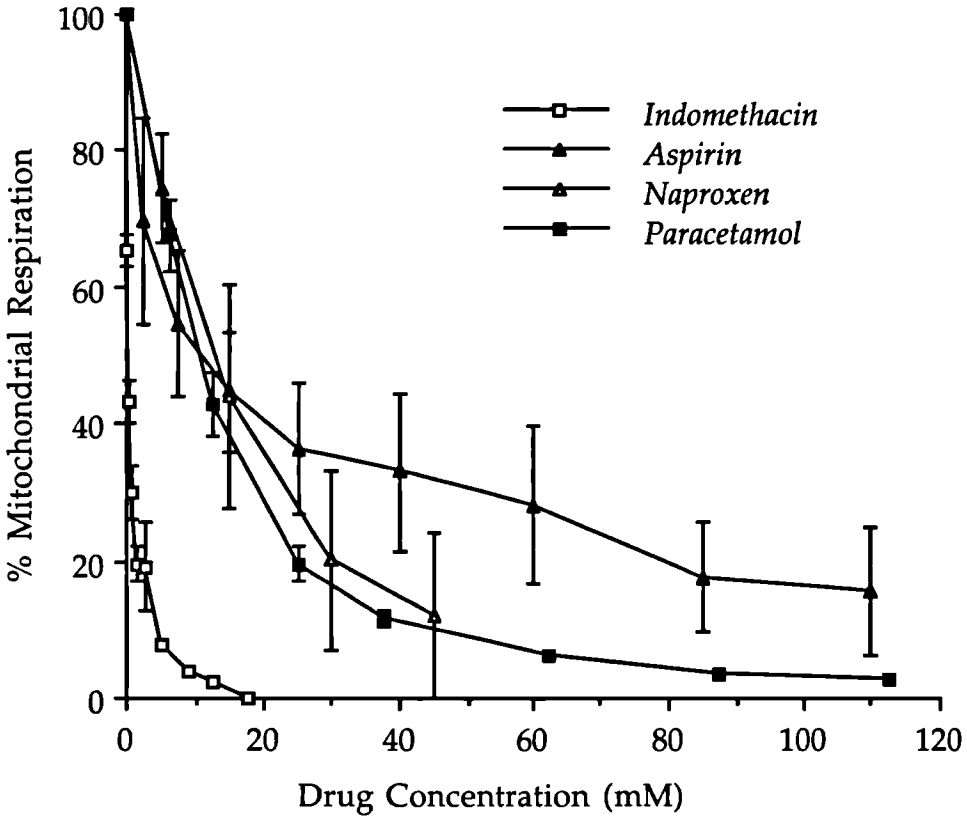


Figure 3.6 Effect of drugs on glutamate/malate (complex I) - stimulated mitochondrial respiration

To a mitochondrial suspension (protein concentration 2.9 to 6.7 mg) was added 10mM glutamate, 10mM malate, 0.1μM FCCP then increasing concentrations of the drugs indomethacin, aspirin, naproxen and paracetamol. Mitochondrial respiration was monitored at 30°C using an oxygen electrode. Results are mean ± SEM of 3 experiments (separate liver preparations). IC_{50} values (mM) were indomethacin = 0.3, aspirin = 11, naproxen = 13 and paracetamol = 10.7. All points differed significantly from control, $P < 0.05$, Student's t-test, except for first measurement at 0 mM of all drugs and second at 2.5mM of aspirin.

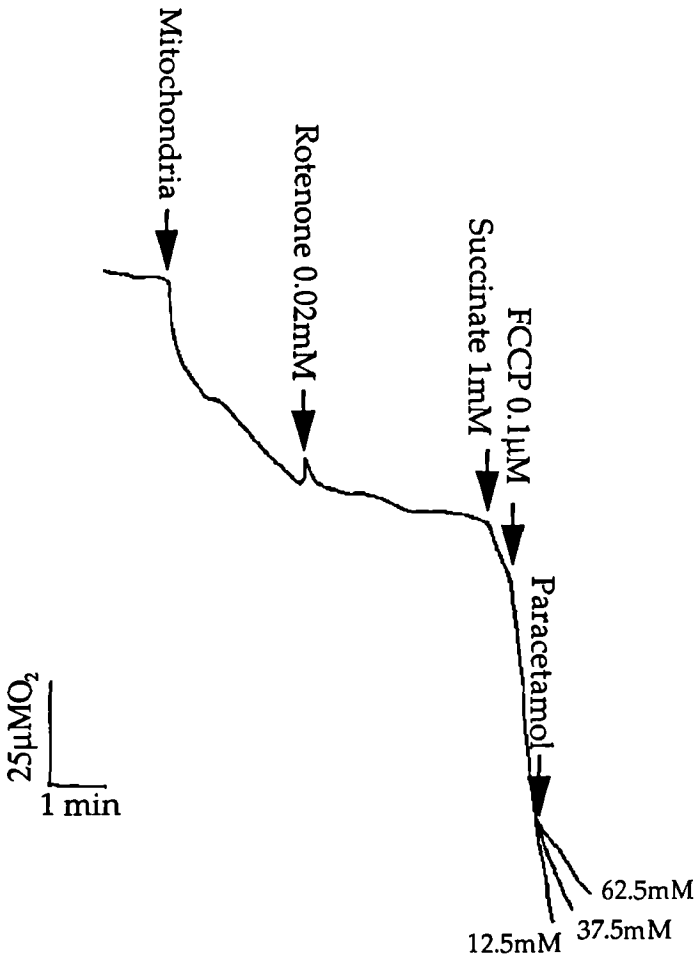


Figure 3.7 Inhibition of complex-II mediated mitochondrial respiration by paracetamol

Oxygen electrode trace showing paracetamol effect on succinate-supported mitochondrial oxygen consumption. Arrows indicate additions made to the mitochondria (2.9 to 6.7 mg protein). Rotenone was added to eliminate electron transport from complex I and FCCP to uncouple the mitochondria.

the oxygen electrode trace. In figure 3.8 effects of all the drugs have been converted to percentage of control. The inhibitory potencies of the drugs according to their IC_{50}'s were:

indomethacin (0.35) › naproxen (7.5) › aspirin (13) › paracetamol (23.9).

3.4.3 Discussion

This study showed that the drugs indomethacin, aspirin, naproxen and paracetamol all concentration-dependently inhibited uncoupled mitochondrial respiration (FCCP-induced state 3 respiration), whether the electrons were being fed in from complex I or from complex II. The IC_{50} of naproxen at inhibiting complex I-mediated respiration was slightly greater than its IC_{50} against complex II-mediated respiration, suggesting perhaps that naproxen was more effective at inhibiting respiration when electrons were being fed into complex II.

Unlike the results of the effects of the various drugs on uncoupling (chapter 2), both the NSAIDs and the non-NSAID paracetamol brought about inhibition. The IC_{50} for paracetamol at inhibiting complex II-mediated respiration was, however, two-fold greater than its IC_{50} at inhibiting complex I-mediated respiration. This would suggest that paracetamol was more effective at inhibiting respiration when electrons were
being fed in from complex I.

The fact that paracetamol inhibits electron transport would, at first, suggest that inhibition of respiration by NSAIDs might not be important in the pathogenesis of NSAID enteropathy. However, an increase in intestinal permeability and inflammation caused by paracetamol cannot be ruled out, as studies investigating these effects have not been done. Indeed, reduced ATP

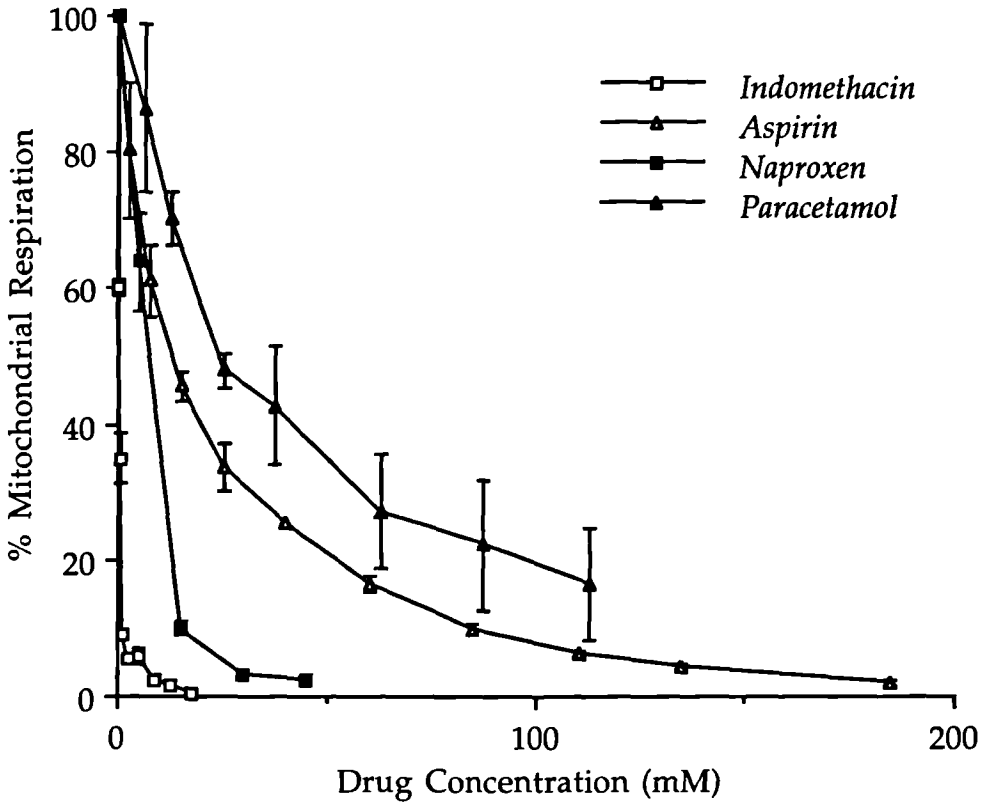


Figure 3.8 Effect of drugs on succinate (complex II) - stimulated mitochondrial respiration

To a mitochondrial suspension (protein concentration 2.9 to 6.7 mg) was added 20μM rotenone, 1mM succinate, 0.1μM FCCP then increasing concentrations of the drugs indomethacin, aspirin, naproxen and paracetamol. Mitochondrial respiration was monitored at 30°C using an oxygen electrode. Each point represents the mean ± SEM of 3 experiments (separate liver preparations). IC_{50} values (mM) were indomethacin = 0.35, aspirin = 13, naproxen = 7.5 and paracetamol = 23.9. All points differed significantly from control, $P < 0.05$, Student's t-test, except for the first measurement at 0 mM of all drugs, and second of aspirin and paracetamol at 2.5mM and 6.25mM respectively.

levels caused by the inhibition of mitochondrial respiration by paracetamol, could possibly bring about such deleterious effects in the intestine, in a similar manner to NSAIDs. In addition, although ulcers are not generally observed with paracetamol, this does not necessarily mean that there is no increase in permeability with this drug. The driving force in converting the intestinal damage to ulcers may be the concomitant inhibition of cyclooxygenase-1. This would effectively reduce the synthesis of mucosal prostaglandins, which are important for the regulation of blood flow, leading to vasoconstriction, ischaemia and eventually ulcer formation (Anthony *et al.*, 1995). Therefore, although paracetamol appears to inhibit the respiratory chain at high concentrations, which might possibly cause an increase in intestinal permeability, the drug is without effect on cyclooxygenase perhaps explaining the lack of ulcer formation.

The following compares the results from other studies with that of the present work.

Glutamate/Malate-Supported Respiration

Table 3.1 compares the present data with those of others using glutamate and malate as substrates. Byczkowski and Korolkiewicz (1977) suggested that indomethacin inhibited the electron transport chain sites before cytochrome *c*. Later, the same authors postulated that the inhibition of state 3 respiration by indomethacin was caused by a collapse of the mitochondrial cationic gradient, since the drug has a higher ionophoretic potency than that for classic uncouplers (Byczkowski and Korolkiewicz, 1978). In the study by Porter and Dawson (1979), on the other hand, the inhibition by paracetamol was shown to be due to partial blockage of mitochondrial electron transport between NADH dehydrogenase and cytochrome *b* .

Table 3.1 The concentrations of the NSAIDs causing 50% inhibition of glutamate/malate-supported respiration : a comparison of the data from this study with those of others

Drugs tested	IC50 (mM)	Mitochondria	Notes	Reference
Indomethacin Aspirin Naproxen Paracetamol	0.3 11 13 10.7	Rat liver	FCCP-induced state 3 respiration	This study
Indomethacin	0.25	Rat liver	DNP-induced state 3 respiration; only glutamate used as substrate.	Byczkowski & Korolkiewicz, 1977
Indomethacin	0.25	Rat liver	ADP-induced state 3 respiration; only glutamate used as substrate	Byczkowski & Korolkiewicz, 1978
Indomethacin	0.04	Rat liver	ADP-induced state 3 respiration	Kawai *et al.*, 1985
Indomethacin	0.2	Rat liver	ADP-induced state 3 respiration	Bãños & Reyes, 1989
Indomethacin	0.24	Guinea pig gastric mucosa	ADP-induced state 3 respiration	Priess & Sewing, 1985
Paracetamol	1.8	Mouse liver	ADP-induced state 3 respiration	Ramsay *et al.*, 1989
Paracetamol	9.7	Rat kidney	DNP-induced state 3 respiration; only glutamate used as substrate	Porter & Dawson, 1979

Mingatto *et al.* (1996) found that 2-4 mM aspirin and 5-7 mM paracetamol inhibited state 3 respiration in rat kidney mitochondria, whether induced by ADP or the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). They noted that the pattern of inhibition by aspirin paralleled that of the respiratory chain inhibitors of complex I (rotenone), complex III (antimycin A), and complex IV (potassium cyanide); since the inhibition of state 3 respiration by aspirin could not be released by CCCP. However, paracetamol inhibition could be released by CCCP; therefore, paracetamol showed a pattern similar to that expressed by the inhibitors of the F_0F_1ATPase (oligomycin) and the ADP/ATP carrier (atractyloside). (The effects of the drugs on succinate oxidation were similar to those mentioned here on glutamate/malate oxidation). In contrast to the present results using aspirin, Petrescu and Tarba (1997) showed that 2mM aspirin failed to inhibit ADP-induced state 3 respiration.

Succinate-Supported Respiration

As with the findings of the present study, Mehlman *et al.* (1972) showed that 0.6 mM aspirin could significantly inhibit DNP-uncoupled respiration up to 66%. Similarly, when state 3 respiration was induced by ADP, 0-0.7 µmoles aspirin/mg protein (approximately 0-2.5 mM) inhibited respiration concentration-dependently by up to 60% of control (Leblondel and Allain, 1979) whilst in another study, aspirin gave an IC_{50} of 4.5mM (Keller *et al.*, 1992). However, Tokumitsu *et al.* (1977) failed to show any inhibition in oxygen uptake with 1mM aspirin, in mitochondria uncoupled with DNP. Perhaps in this case, a higher drug concentration may have inhibited, as in the present study.

Nevertheless, Tokumitsu *et al.* (1977) did find that 0.1 mM indomethacin inhibited oxygen uptake to 33% of control. Likewise, Priess and Sewing (1985)

showed that indomethacin inhibited ADP-induced state 3 respiration in guinea pig gastric mucosal mitochondria, giving an IC_{50} of 0.18mM. These authors postulated the site(s) of inhibition in the vicinity of complexes I and III of the respiratory chain. Byczkowski and Korolkiewicz (1977) reported an IC_{50} of 0.3mM indomethacin of DNP-uncoupled respiration in rat liver mitochondria. Again in rat liver mitochondria, both Kawai *et al.* (1985) and Baños and Reyes (1989) also demonstrated inhibition with indomethacin, but when state 3 respiration was stimulated with ADP. In the former study, the IC_{50} was estimated to be > 0.2mM. In the latter study, naproxen was also studied and 0.3 mM inhibited respiration to only 80% of control, contrary to the present findings. Baños and Reyes (1989), however, failed to explain why amongst the 10 NSAIDs they studied, naproxen appeared to be alone in its anomalous effects on rat liver mitochondrial oxygen uptake.

In contrast to their findings with glutamate/malate-supported respiration, both Ramsay *et al.* (1989), using mouse liver mitochondria, and Porter and Dawson (1979), using rat kidney mitochondria, found no inhibition by paracetamol when succinate was used as the substrate. This would suggest that paracetamol preferentially blocks electron transport at complex I, though this has not been studied further.

Overall, there appeared to be a close correlation between the present study and others when comparing the relative potency of the drugs at inhibiting mitochondrial respiration. Although the actual IC_{50} values varied amongst the different studies, this could have been due to, for example, different experimental conditions especially problems with drug solubility. These compounds being lipophilic may have, in some cases, been used in suspension and not in solution. Thus, the effective drug concentration per mg mitochondrial protein would possibly be less than previously estimated.

Other NSAIDs including diflunisal and diclofenac, have also been found to inhibit ADP-driven state 3 respiration in a concentration-dependent manner when glutamate plus malate or when succinate were used as substrates (McDougall *et al.*, 1983; Baños and Reyes, 1989).

3.5 Ferricyanide Studies

Studies on electron transport in isolated mitochondria can be carried out using terminal electron acceptors other than oxygen, enabling shorter sections of the respiratory chain to be studied. One of most extensively used of such reagents is potassium ferricyanide (Caswell and Pressman, 1969), an artificial electron acceptor which can draw off electrons from specific sites in the respiratory chain. Ferricyanide, together with respiratory inhibitors, can be used to determine both the sequence of electron carriers and also the effects of various compounds on the individual respiratory complexes. The use of ferricyanide as an artificial electron acceptor is preferred since it is able to operate at substrate concentrations with little deleterious effects to the mitochondria.

3.5.1 Materials and Methods

Principle of the Assay

On accepting an electron, ferricyanide is reduced to ferrocyanide. Ferricyanide possesses an absorption peak at 420 nm, which on ferricyanide reduction disappears (Pressman, 1955). Thus, it is possible to monitor the rate of ferricyanide reduction spectrophotometrically (figure 3.9).

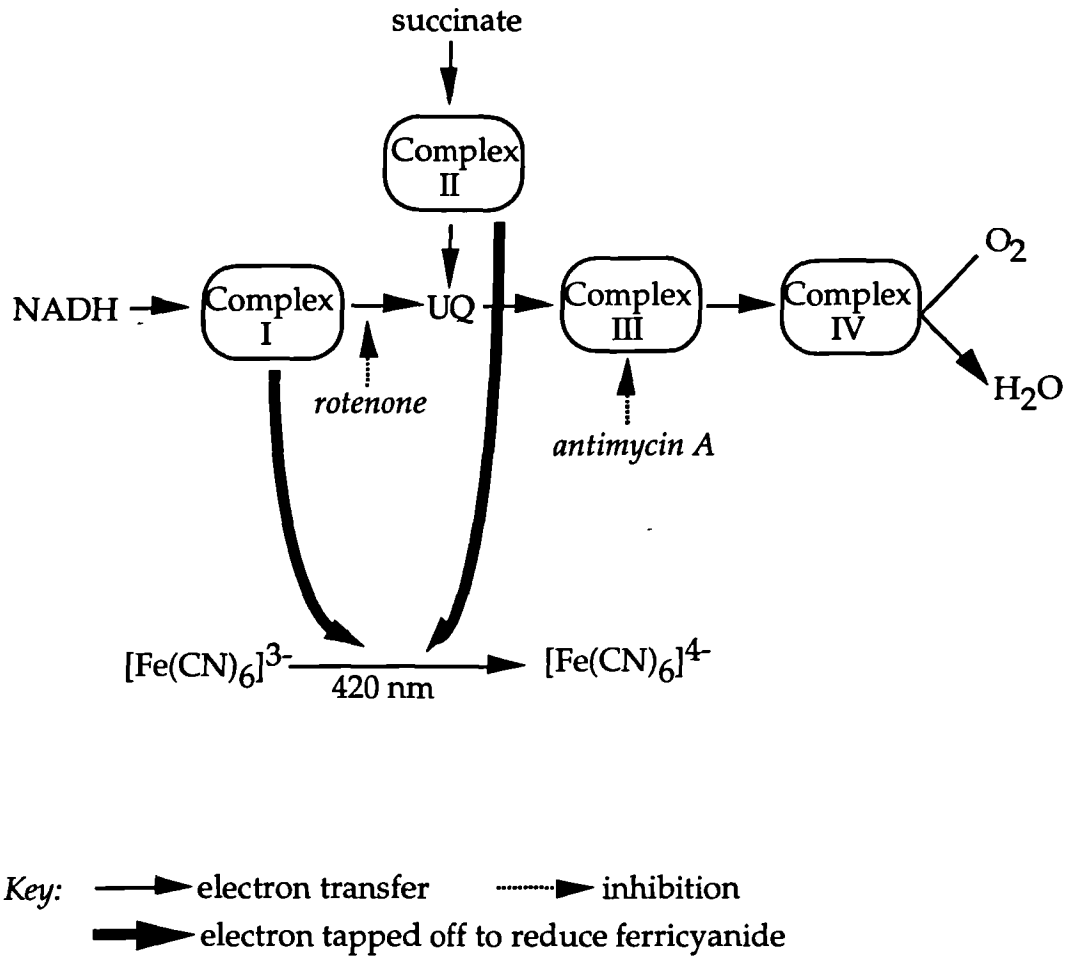


Figure 3.9 Ferricyanide assay system

Schematic representation of electron transport chain. To assay complex I, the substrates NADH, glutamate, malate and the inhibitor rotenone were used, and electrons were drawn off complex I to reduce ferricyanide. For complex II, the substrate succinate in the presence of the inhibitors antimycin A and rotenone were used, and electrons were tapped off from complex II to reduce ferricyanide. Ferricyanide reduction was measured spectrophotometrically at 420 nm.

Ferricyanide can not penetrate the inner mitochondrial membrane (IMM) (Mitchell and Moyle, 1969). In intact mitochondria the major site of ferricyanide reduction is cytochrome *c* which is located in the outer face of the IMM (Klingenberg, 1970). Therefore, the mitochondria need to be sonicated for the assay, to enable access of ferricyanide to the matrix-facing side of the IMM.

Assay of Electron Transport from Complex I to Ferricyanide

Isolated rat liver mitochondria were sonicated for 5 to 10 minutes using a pulse sonicator (Branson) at power setting 3. 100µl sonicated mitochondria was added to 3ml of a reaction mixture containing 2.5mM potassium ferricyanide dissolved in: 150mM sucrose, 10mM potassium chloride, 5mM magnesium chloride and 1mM potassium dihydrogen phosphate (pH 7.0). In order to study electron transfer specifically from complex I, 2mM NADH, 1mM glutamate and 1mM malate (complex I substrates), and 0.1 mM rotenone (complex I inhibitor), were included in the reaction mixture. Ferricyanide reduction was determined by measuring the change in absorbance at 420 nm. NSAIDs indomethacin, aspirin and naproxen were added sequentially at different concentrations, as was the analgesic paracetamol.

Assay of Electron Transport from Complex II to Ferricyanide

Assay carried out as above, with the exception that 1mM succinate, 1mM rotenone and 1µM antimycin A were included in the reaction mixture, to assay electron transport specifically from complex II to ferricyanide.

3.5.2 Results

Figures 3.10 and 3.11 show the effect of the NSAIDs indomethacin, aspirin and naproxen on electron transport from complex I and complex II respectively. From these figures it can be seen that all the NSAIDs concentration-dependently inhibited both complex I and complex II-mediated electron transport. However, the non-NSAID paracetamol did not inhibit electron transport from either complex (results not shown). The estimated IC_{50}'s (mM/mg protein) for the NSAIDs for complex I and complex II-mediated electron transport were:

Drug	Complex I	Complex II
Indomethacin	0.21	0.3
Aspirin	0.47	3.8
Naproxen	0.43	1.1

3.5.3 Discussion

The present results show that indomethacin was the most potent drug in terms of requiring the least concentration to substantially inhibit ferricyanide-dependent electron transport from both complex I and II. This is in accordance with the previous findings using the oxygen electrode (section 3.3). In addition, indomethacin seemed to be more effective at inhibiting complex II-mediated electron transport, as seen by its lower IC_{50} for complex II. However, the other two NSAIDs aspirin and naproxen had significantly higher IC_{50}'s for complex II, suggesting that they are less effective at inhibiting electron transport from complex II than from complex I. The use of sonicated mitochondria for the ferricyanide-dependent electron transfer assay,

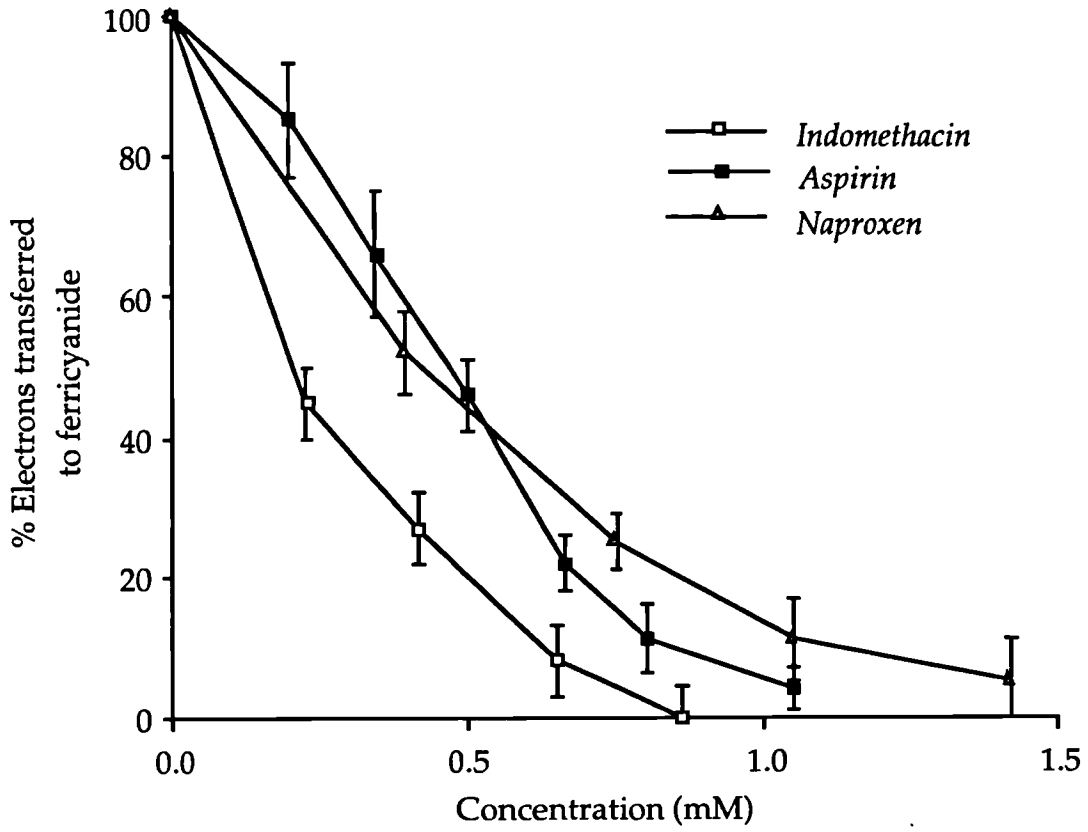


Figure 3.10 Effect of NSAIDs on ferricyanide-dependent electron transport from complex I

To sonicated mitochondria suspended in a reaction mixture containing potassium ferricyanide (see main text), was added 2mM NADH, 1mM glutamate, 1mM malate and 0.1mM rotenone. The rate of complex I-mediated ferricyanide reduction was monitored spectrophotometrically at 420nm. Increasing concentrations of the NSAIDs indomethacin, aspirin and naproxen were then added to observe their effects on complex I-mediated ferricyanide reduction. Each point is the mean of 3 experiments ± SEM (3 separate rat liver preparations). Except for the first measurement at 0mM, all points were significantly different from control, $P < 0.05$, Student's t-test.

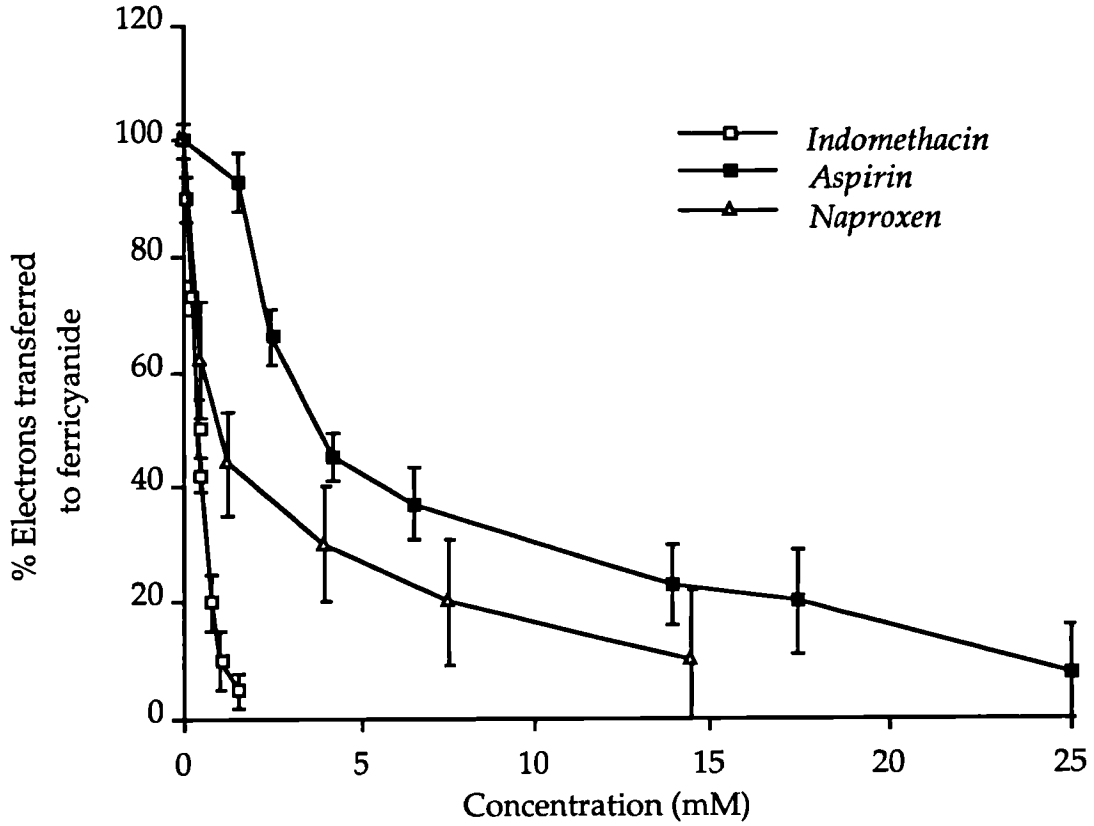


Figure 3.11 Effect of NSAIDs on ferricyanide-dependent electron transport from complex II

To sonicated mitochondria suspended in a reaction mixture containing potassium ferricyanide (see main text), was added 1mM succinate, 1mM rotenone and 1μM antimycin A. The rate of complex II-mediated ferricyanide reduction was monitored spectrophotometrically at 420nm. Increasing concentrations of the NSAIDs indomethacin, aspirin and naproxen were then added to observe their effects on complex I-mediated ferricyanide reduction. Each point is the mean of 3 experiments ± SEM (3 separate rat liver preparations). Except for the first measurement at 0mM for all the drugs and the second measurement of indomethacin at 0.025mM and aspirin at 1.5mM, all points were significantly different from control, $P < 0.05$, Student's t-test.

eliminates the possibility that the inhibitory effect of the drugs may have been on substrate transport into the matrix.

The fact that paracetamol did not inhibit electron transport, in contrast with the findings of the oxygen electrode studies, suggest that paracetamol inhibition of electron transport occurs beyond complexes I and II in the respiratory chain, i.e. at complex III or IV or that paracetamol inhibits substrate entry.

In summary, all the NSAIDs studied inhibited ferricyanide-dependent electron transport. This has not previously been reported. Despite the different IC_{50} values, the range of potencies was similar for complex I and complex II-mediated electron transport:

indomethacin > aspirin > naproxen.

3.6 EPR Studies

Present within each of the complexes of the respiratory chain are multiple forms of iron-sulphur (Fe-S) clusters which can be detected by electron spin/paramagnetic resonance (ESR/EPR). Figure 3.12 shows the electron transport system in more detail, with the components of complexes I to IV. As can be seen from this figure, complex I contains, along with its other prosthetic group flavomononucleotide (FMN), five separate Fe-S clusters, termed FeS_{N1a}, FeS_{N1b}, FeS_{N2}, FeS_{N3} and FeS_{N4} according to the nomenclature of Ohnishi (Ohnishi, 1979). Likewise, complex II has together with its other prosthetic groups flavin adenine dinucleotide (FAD) and cytochrome *b560*, three Fe-S centres known as FeS_{S1}, FeS_{S2} and FeS_{S3}. Complex III has only one Fe-S cluster, known as the Rieske centre after the

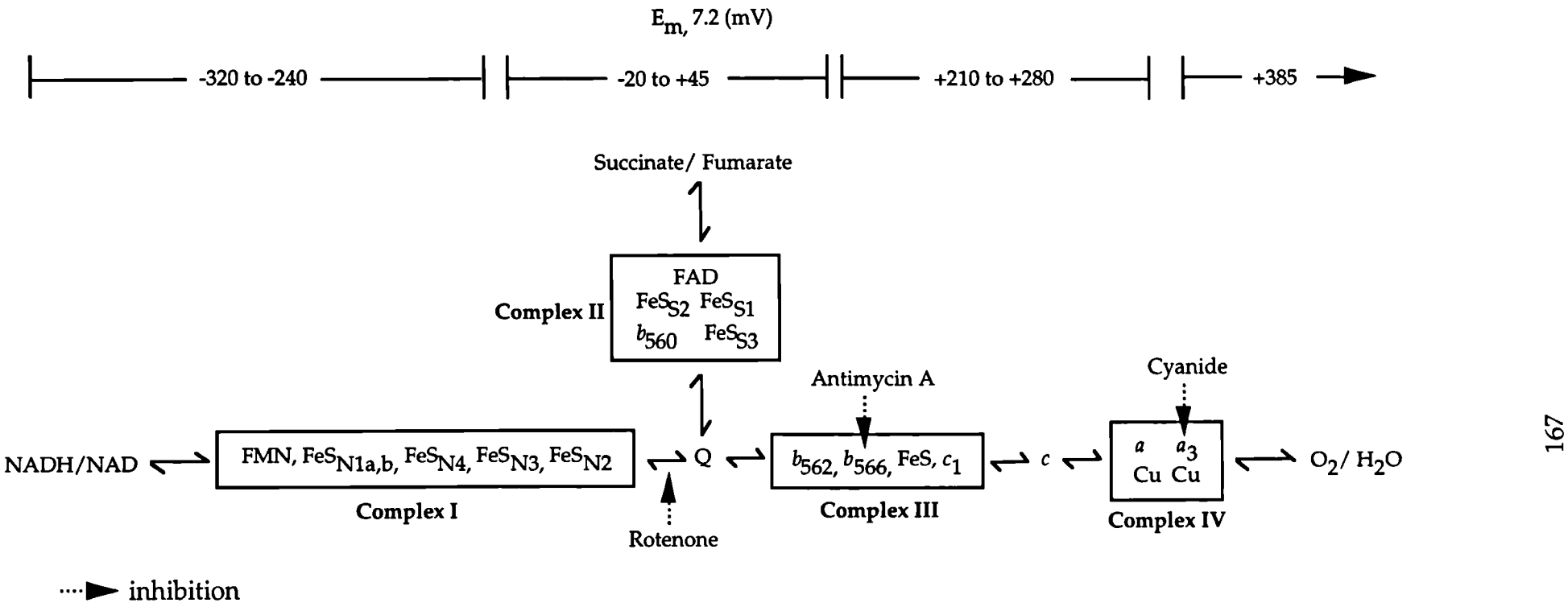


Figure 3.12 Profile of the mitochondrial electron transport system

This diagram shows the well characterised components of complexes I, II, III and IV. Only EPR-detectable iron-sulphur (FeS) clusters are shown. FeS subscripts denote the individual FeS clusters according to the nomenclature of Ohnishi (Ohnishi and Salerno, 1982). *a*, *b* and *c* are cytochromes *a*, *b* and *c* respectively (subscripts on *b* cytochromes denote position of their α peaks at ambient temperatures). For other abbreviations see text. Adapted from Hatefi (1985).

person who discovered it (Rieske *et al.*, 1964). Although complex IV has no Fe-S centres, it does contain EPR-detectable iron from the haem centre of cytochrome oxidase (also known as cytochrome aa_3).

The respiratory chain contains 3 types of Fe-S clusters each with a different ratio of iron to sulphur; these are the [2Fe-2S], the [4Fe-4S], and the [3Fe-4S] clusters (figure 3.13). For the last two types of cluster, the Fe atoms are each coordinated by four acid-labile S atoms arranged tetrahedrally around them. The majority of the Fe-S clusters are coordinated by four cysteine residues from the protein backbone of the respiratory complex. However, in the Rieske-type centre, the ligands are 2 cysteines and 2 histidines. The iron atom in these clusters undergoes oxidoreduction between the reduced (Fe^{2+}) and oxidised (Fe^{3+}) states, enabling the proteins to function as one-electron carriers.

3.6.1 The Theory of EPR

There is extensive and detailed literature on the theory of EPR, which is summarised well by Beinert and Palmer (1965). For the purpose of this thesis the rationale of EPR spectroscopy can be simplified.

The majority of stable molecules are held together by bonds in which electron spins are paired; in this situation there is no net electron spin, no electronic magnetic moment, and hence no interaction between the electron spins and an applied magnetic field. Some atoms and molecules, however, do contain one or more electrons with unpaired spins, and these molecules exhibit electron spin resonance (ESR). Since the molecules detected are paramagnetic, this type of spectroscopy is often termed electron paramagnetic resonance (EPR). EPR spectroscopy essentially detects the transitions between two energy

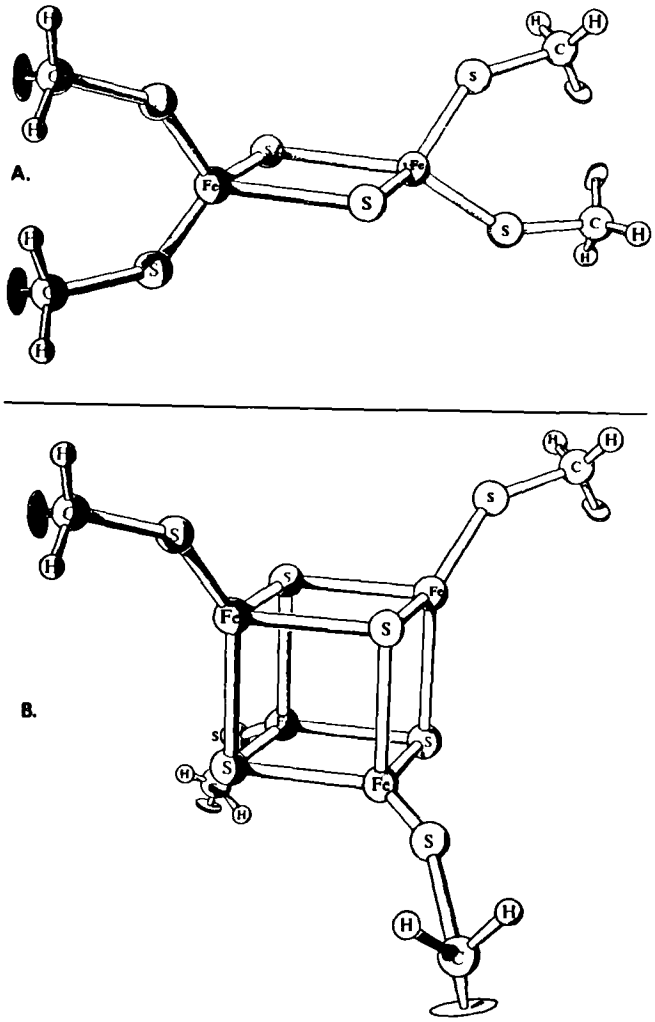


Figure 3.13 3D chemical structure of iron-sulphur clusters
A. [2Fe-2S] B. [4Fe-4S]
Source: McGilvery (1979).

levels, induced by the application of an external magnetic field and an appropriate frequency radiation.

EPR spectra are recorded as a series of peaks and troughs of characteristic shapes with characteristic g values (g is a spectroscopic splitting factor). In principal, the relative intensities of the signals are proportional to the relative number of unpaired electrons in the system (Ebsworth *et al.*, 1987). The temperature at which EPR spectra are measured can be important. For Fe-S centres, low temperature EPR, as opposed to EPR at ambient (room) temperatures, results in increased sensitivity, an improved signal-to-noise ratio, making quantitative measurements more reproducible. In contrast, some free radicals are detected more readily at room temperature.

With the exception of the S3 Fe-S cluster of complex II, all the other Fe-S clusters of respiratory complexes I to III have paired electrons and are therefore "diamagnetic". However, in order to be detected by EPR, they need first to be made paramagnetic by one-electron reduction so that they contain an unpaired electron. In the reduced state, these Fe-S centres exhibit an EPR spectrum at low temperature that is characterised by a prominent signal (g_y) with maximal absorption at g=1.85 to g=1.95, and two lower intensity features (g_x and g_z). Table 3.2 summarises the properties of the Fe-S centres of the respiratory chain, as found by EPR.

3.6.2 Aims of the EPR Studies

The aim of the studies in this section was to see if any of these components of the respiratory chain were affected by the NSAIDs, which could explain the inhibitory properties of these drugs. The appearance or disappearance of EPR signals, or a decrease in the peak-to-trough (PTT) value when compared to

Table 3.2 **Properties of the iron-sulphur clusters of the respiratory complexes**

Complex	Cluster	*g*-positions			Fe:S type
		g_x	g_y	g_z	
I	FeS_{N1a}	1.91	1.95	2.03	[2Fe-2S]
I	FeS_{N1b}	1.92	1.94	2.02	[2Fe-2S]
I	FeS_{N2}	1.92	1.92	2.05	[4Fe-4S]
I	FeS_{N3}	1.86	1.93	2.04	[4Fe-4S]
I	FeS_{N4}	1.87	1.93	2.10	[4Fe-4S]
II	FeS_{S1}	1.91	1.93	2.03	[2Fe-2S]
II	FeS_{S2}	1.91	1.93	2.03	[2Fe-2S]
II	FeS_{S3}*	1.99	2.01	2.02	[3Fe-4S]
III	FeS_{III}**	1.81	1.89	2.025	[2Fe-2S]

* EPR active in oxidised state; rest are EPR active in reduced state. ** Also known as the "Rieske" centre. Based on data from Orme-Johnson *et al.* (1974) and Ohnishi (1979).

control, were used as indices of drug effect. The control was the solvent, 10% (v/v) DMSO.

The experiments were carried out using beef heart submitochondrial particles (SMP), because these contain higher concentrations of redox proteins in the inner mitochondrial membrane than isolated rat liver mitochondria, and the corresponding EPR signals were found to be greater. SMP's prepared from sonically disrupted mitochondria essentially consist of sealed inner mitochondrial membrane vesicles, where the IMM has been turned inside out. In addition, the matrix enzymes are removed so only signals from the

membrane-associated respiratory complexes are seen. An added advantage is that the substrate NADH, which reduces complex I but cannot cross the IMM, can now be used as a reductant.

The experiments were carried out first on oxidised SMP's (as prepared), in order to detect the EPR signals from centre S3 of complex II, and Cu_A of cytochrome oxidase, and then in the reduced state to study the remaining Fe-S clusters. Although there are two centres $N1_a$ and $N1_b$, only one of these centres is usually detected, and is termed centre N1.

As with the oxygen electrode and ferricyanide inhibition experiments, specific substrates were used to reduce the individual complexes, to study each complex in isolation. Dithionite was used to reduce the sample, in order record an EPR spectrum of all the centres, except centre S3 which is EPR-silent when reduced. NADH was used to reduce complex I and succinate was used for complex II.

With all the experiments the temperature was varied from 8K to 40K. This is because different signals become apparent at different temperatures, summarised as:

Temperature (helium)	Fe-S clusters observed by EPR
8 to 15 K	[4Fe-4S]; [3Fe-4S]
20 to 77 K	[2Fe-2S]
40 K	Cu_A^{3+} of cytochrome oxidase; Rieske

3.6.3 Materials and Methods

Materials

Beef hearts were provided by the University of Essex, Essex, England. Sorbitol, trypsin, trypsin inhibitor, disodium adenosine triphosphate (Na_2ATP), reduced nicotinamide adenine dinucleotide (NADH) were supplied by the Sigma Chemical Company Ltd., Dorset, U.K. N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) was supplied by Fluka Chemicals, Dorset, England.

Isolation of Beef Heart Mitochondria

A modification of the protocol described by Rickwood *et al.* (1987) for the isolation of rat liver mitochondria, was used for isolating coupled mitochondria in large scale from bovine heart tissue. This modified procedure is particularly suited for EPR studies.

Fat and connective tissue was removed from fresh hearts, and the muscle minced finely. The mince was suspended in an equal volume of a buffer containing 50mM EDTA, 10mM Tris, 210mM sorbitol, 70mM sucrose, 0.1%

BSA, pH 7.6 (high-EDTA buffer), and washed with a minimum of 6 changes to remove blood and fat impurities.

The tissue was incubated with trypsin (0.5mg/g tissue) for 30 minutes, to aid digestion of the tough connective tissue that prevents successful homogenisation. To prevent further digestion, trypsin inhibitor (1.5mg/g tissue) was added to the incubation, and the preparation washed with several changes of high-EDTA buffer. The tissue was homogenised, a little at a time, using a MSE Atomix blender at medium speed, with two 5 second bursts interrupted by 30 second intervals. The homogenate was distributed into pre-cooled 500ml Sorvall centrifuge tubes and centrifuged at 1,500g for 5 minutes.

The supernatant was decanted off carefully and filtered through four layers of muslin to remove fat. The filtrate was centrifuged at 13,000g for 10 minutes, and the resultant dark-brown mitochondrial pellet washed and resuspended in a buffer comprising of 100µM EDTA, 10mM Tris, 225µM sorbitol, 75mM sucrose, 0.1% BSA, pH 7.6 (low-EDTA buffer). This material was recentrifuged as above at 13,000g and the final pellet resuspended in low-EDTA buffer. The prepared sample was kept frozen at 77K until required.

Preparation of Beef Heart Submitochondrial Particles

The beef heart mitochondria (BHM) was diluted to a final concentration of 10mg protein/ml in 10mM HEPES, 1mM $MgCl_2$, 1mM ATP, 5mM succinate, 0.2mM NADH and 1% BSA, pH 7.1 (Medium A). The mitochondrial suspension was centrifuged at 26,000g for 10 minutes at 4°C and the supernatant decanted. The pellet was resuspended in Medium A and sonicated in 3ml aliquots on ice, with eight 15 second bursts, interrupted by 15 second intervals, at 18 amps, using a microtip probe. The sonicated suspension was then centrifuged at 26,000g for 10 minutes at 4°C.

The supernatant was removed and kept on ice whilst the remaining mitochondrial pellet was again resuspended in Medium A, then sonicated and centrifuged as before. The supernatants were combined and spun in a Pegasus Ultracentrifuge at 130,000g for 1 hour at 4°C.

The supernatant was removed and the remaining red-brown pellet was resuspended in 40ml of 250mM sucrose and 50 mM HEPES, pH 7.5, and recentrifuged at 130,000g for 1 hour. The supernatant was again discarded and the final pellet, containing the submitochondrial particles, was resuspended in a minimal volume of the sucrose solution.

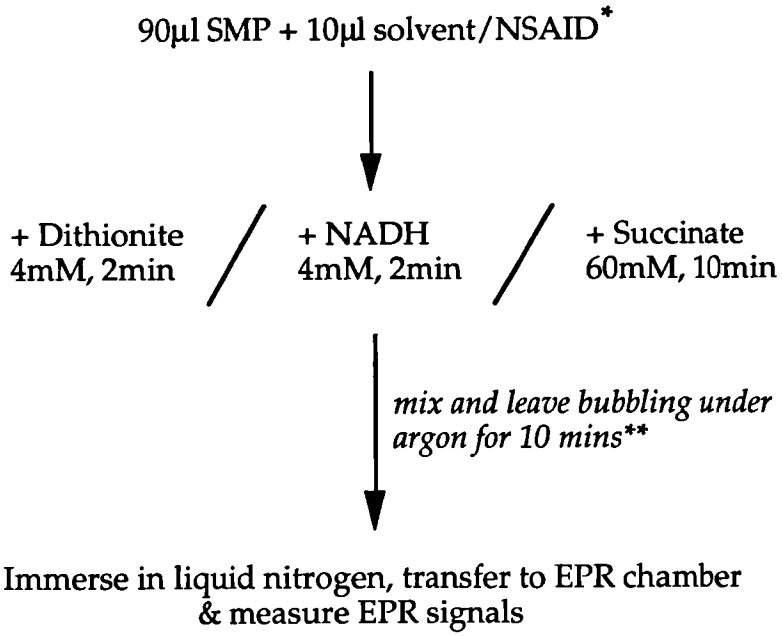
Sample Preparation for EPR

Oxidised samples

10µl of solvent or NSAID was added to 90µl SMP in a quartz EPR tube (3mm internal diameter). For measurement under oxidised conditions, mixing of the sample on the bench for approximately 1 minute was sufficient. The sample was frozen by gradually immersing the EPR tube into liquid nitrogen (-70°C), and stored until required for measurement.

Reduced samples

Preliminary experiments were performed to find out how much reductant was required to give optimum reduction of the respiratory complexes. The general sequence of additions was:



*The final concentrations of the NSAIDs added were: 25mM indomethacin, 50mM aspirin and 100mM naproxen. These were the minimum concentrations required to show an effect, based on preliminary experiments. The solvent was 10% DMSO or distilled water and preliminary experiments showed that the EPR signals were unaffected by these solvents.

**Argon gas was introduced into the EPR tube just above the level of the mixture, in order to exclude atmospheric oxygen from the system.

EPR Measurement

A Bruker ESP 300 spectrometer fitted with an ESR900 liquid helium flow cryostat (Oxford Instruments) was used to carry out EPR measurements.

X-band EPR spectra of the samples were recorded at various temperatures.

3.6.4 Results and Discussion

Oxidised Samples

8K : wide scan

This wide scan spectrum (figure 3.14) reveals the complex II centre S3 (at 3,400G or g=2.02), g=6 signal from haem-Fe^{3+}, and g=4.3 signal from mononuclear non-haem Fe^{3+}. The presence of all these signals in the control indicates that this mitochondrial sample is undamaged by the preparation procedure. No significant change was observed in the low field signals on addition of the solvent 10% DMSO or of the NSAIDs. However, this may be due to low spectral resolution in the g=2 region.

8K : narrow scan

This spectra (figure 3.15) focuses on the most prominent peak observed when BHM SMP's are viewed by EPR as prepared. This signal corresponds to the [3Fe-4S] centre (centre S3) of complex II. From this figure, it can be seen that the NSAIDs indomethacin, aspirin and naproxen, decreased the EPR signal peak size of centre S3. The PTT values were (mm):

Drug	PTT (mm)
Control	52
Indomethacin 25mM	39
Aspirin 50mM	33
Naproxen 100mM	43

Such an effect was also seen *in vivo* in rat intestinal homogenates treated with indomethacin for 1 hour (Somasundaram *et al.*, in preparation). This would initially suggest that the NSAIDs may bind to complex II of the respiratory chain. However, the fact that *in vitro* 10% DMSO also decreased the signal intensity of centre S3, would indicate that the decrease is either a

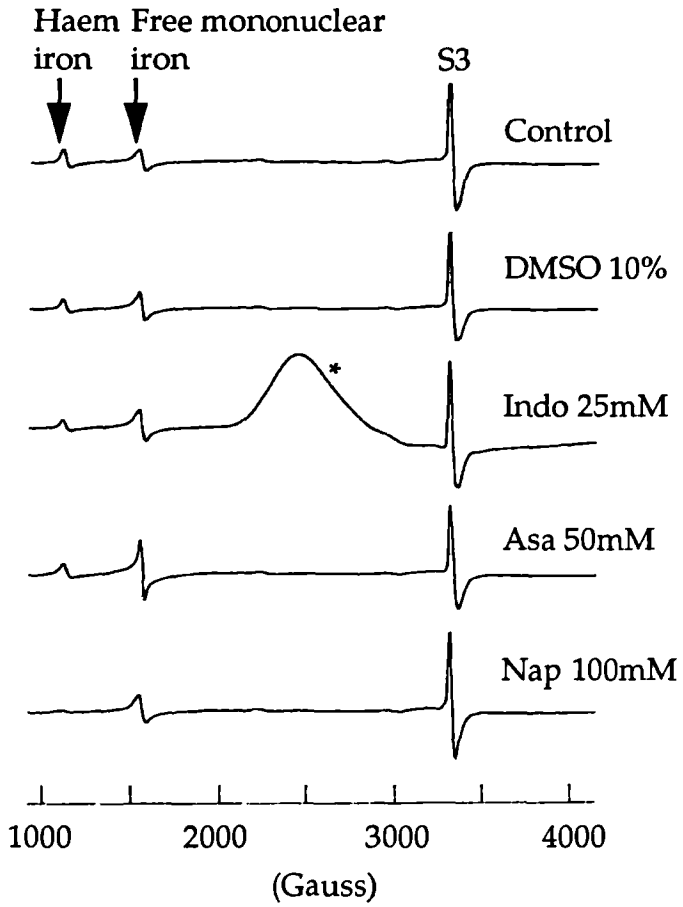


Figure 3.14 **Wide scan EPR trace of oxidised BHM at 8K**

EPR was performed at 8K on BHM as prepared (i.e. oxidised) which was either untreated (control) or incubated with solvent (DMSO), indomethacin (indo), aspirin (asa), or naproxen (nap), at the concentrations shown. See main text for experimental details. S3 = [3Fe-4S] cluster of complex II; * = artefact due to dust contamination. This trace represents an average of 4 scans performed on a single BHM sample.

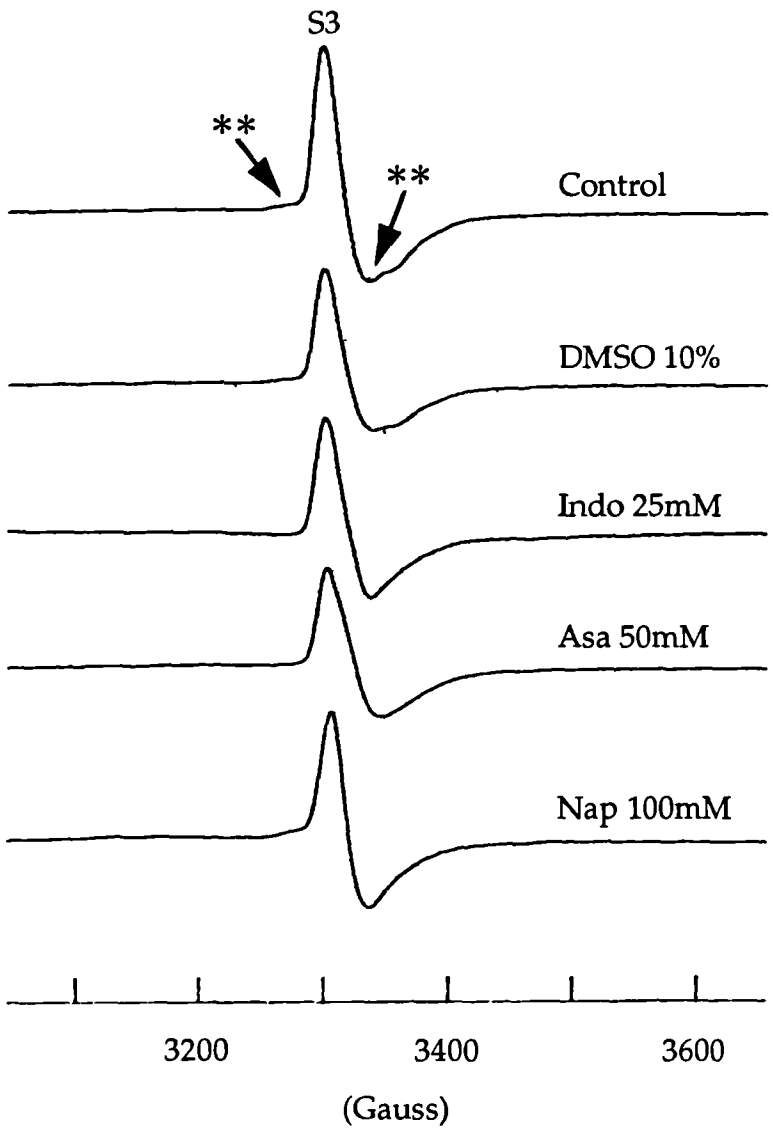
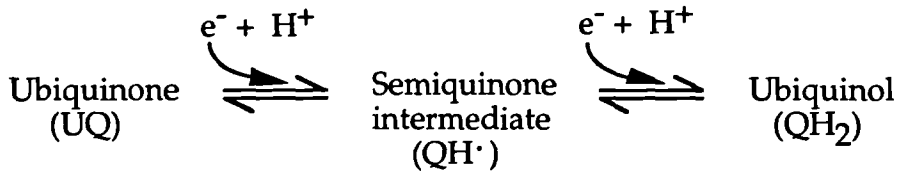


Figure 3.15 EPR trace of oxidised BHM at 8K

EPR was performed at 8K on oxidised BHM which was either untreated (control), or incubated with solvent (DMSO), indomethacin (indo), aspirin (asa) or naproxen (nap) at the concentrations shown. See main text for experimental details. S3 = [3Fe-4S] of complex II; ** = ubiquinone interaction with centre S3. This trace represents an average of 4 scans performed on a single BHM sample.

solvent effect or due to dilution of the sample, since the treated sample was 90µl SMP + 10µl drug/solvent, whereas the control contained 100µl SMP.

Also observed in this spectra were 'wings' on either side of the EPR signal from centre S3 were observed. During electron transfer, reduction of ubiquinone via complex I or II, involves a semiquinone intermediate:



The 'wings' correspond to a spin-spin interaction of the S3 centre with ubiquinone, (i.e. as electrons are transferred from complex II to ubiquinone). The wings were abolished by indomethacin, aspirin and naproxen but not by 10% DMSO. This is, therefore, most likely an NSAID-induced effect. These drugs may be exerting their action by either reducing or oxidising the semiquinone, or by interfering with the reaction of centre S3 with ubiquinone.

40K

At a higher temperature of 40K, the EPR spectrum (figure 3.16) exhibited by the control is typical of Cu_A^{2+} of cytochrome oxidase. 10% DMSO revealed a similar, but slightly decreased, signal, again possibly due to dilution. 50 mM aspirin also seemed to have no effect on the EPR signal. With 100 mM naproxen, a nitrosyl NO signal (at g=2.04) was seen and with 25 mM indomethacin treatment there appeared to be a free radical.

NO reacts with a variety of metal complexes to form high affinity, paramagnetic nitroso complexes. Therefore, NO can be specifically measured by EPR spectroscopy (Xie *et al.*, 1996). These metal-containing proteins, in the

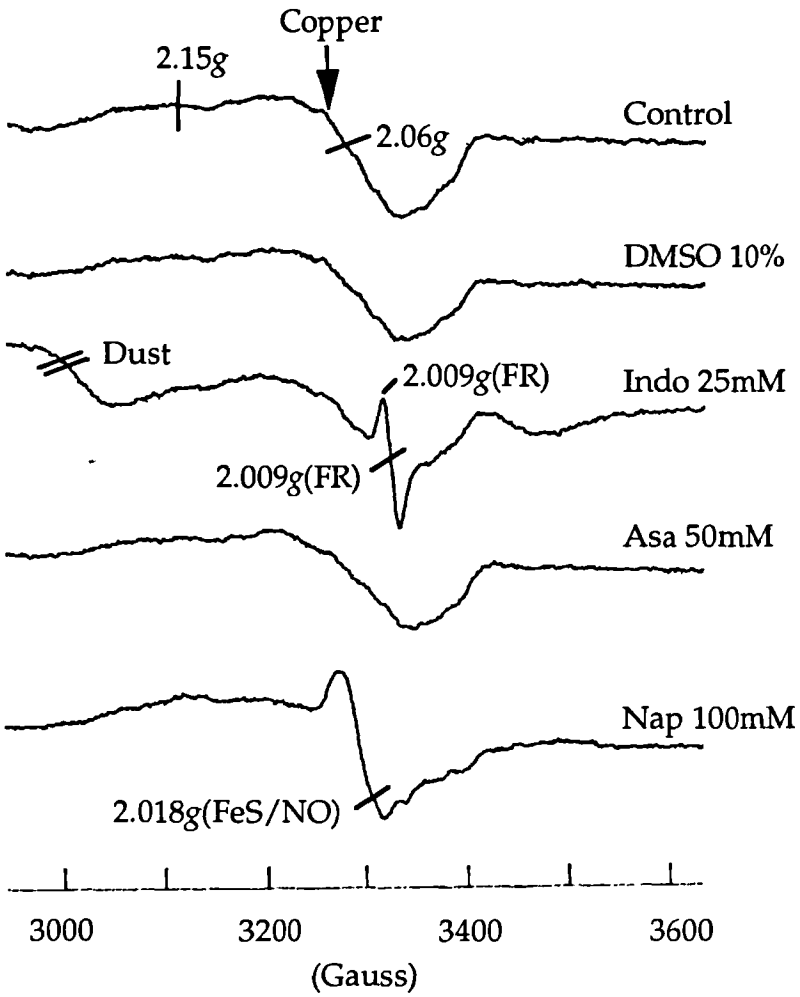


Figure 3.16 **EPR trace of oxidised BHM at 40K**

EPR was performed at 40K on oxidised BHM which was either untreated (control), or treated with solvent (DMSO), indomethacin (indo), aspirin (asa) or naproxen (nap) at the concentrations shown. See main text for experimental details. *g* = spectroscopic splitting factor; FR = free radical; FeS/NO = nitroxyl complex with FeS cluster. This trace represents an average of 4 scans performed on a single BHM sample.

main, are iron-containing proteins such as haem proteins and those with iron-sulphur complexes (e.g. the respiratory complexes). Indeed, the inhibition of mitochondrial respiration seen with NO (see section 2.7, chapter 2) is thought to be via nitrosylation of the iron-sulphur centres of complexes I and II, of aconitase (a key enzyme of the Krebs cycle) (Lancaster and Hibbs, 1990), and by interacting with the haem group of cytochrome *c* oxidase (Cleeter *et al.*, 1994).

However, the question arises as to how NO appeared in the beef-heart SMP's in the presence of naproxen. In biological systems, NO is synthesized by NO synthase (NOS) of which, until recently, there was thought to be three isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) (Venema *et al.*, 1997). (Induction of iNOS by cytokines has been implicated in the pathogenesis of a number of inflammatory diseases, including inflammatory bowel diseases and arthritis (Moncada *et al.*, 1991)). The cellular location for nNOS and iNOS is cytosolic (Marletta, 1994), whereas eNOS is membrane-associated (Busconi and Michel, 1993).

The presence of NOS within mitochondria has only recently come to light. Using isolated rat liver mitochondria, the existence of a functional NOS was shown, associated with the inner mitochondrial membrane but not with the mitochondrial matrix (Ghafourifar and Richter, 1997). NOS activity was detectable in both intact mitochondria and SMP's. Interestingly, this mitochondrial NOS (mtNOS) exerted considerable control over mitochondrial respiration, probably by virtue of its close proximity to the respiratory complexes. So perhaps in the present study, naproxen activates mtNOS to produce NO.

Importantly, NO has also been demonstrated to stimulate the production of the radical superoxide ($O_2^{-\cdot}$), a by-product of mitochondrial respiratory electron transport, catalysed by NOS in the presence of certain substrates and cofactors (Pfeiffer *et al.*, 1997).

Activation of mtNOS ⟶ NO ⟶ $O_2^{-\cdot}$

This, however, is not very surprising considering that other mitochondrial inhibitors such as rotenone, antimycin A and cyanide, also increase the production of $O_2^{-\cdot}$ (believed to be within complexes I and III) (Knowles, 1997). Perhaps, therefore, the results seen with indomethacin in the present study, was a result of activation of mtNOS, producing NO then, in turn, a superoxide radical. Indeed, oxygen-derived free radicals have been implicated in the pathogenesis of NSAID gastropathy (Del Soldato *et al.*, 1985; Pihan *et al.*, 1987; and Vaananen *et al.*, 1991), although other reports have suggested that NSAIDs behaved as free radical scavengers as part of their antiinflammatory mechanism (Bodaness and Chan, 1980; Hiller and Wilson, 1983; Sagone Jr. and Husney, 1987). Maybe in the present study, the concentration of naproxen used was not cytotoxic enough to activate mtNOS beyond the formation of NO. However, this still fails to explain why aspirin had no apparent effect on mtNOS.

Nevertheless, as with the present study, the appearance of a haem-nitric oxide signal by EPR was previously seen *in vivo* in rat jejunal homogenates following indomethacin treatment for 24 hours (Somasundaram *et al.*, in preparation). Pretreatment with an inhibitor of inducible NO synthase, 2,4-diaminohydroxy pyrimidine (DAHP), decreased the intensity of this NO signal significantly. DAHP also prevented indomethacin-induced ulcer formation but did not significantly affect intestinal permeability. These findings suggest that during NSAID enteropathy, NO is released which is somehow involved in the later stages of pathogenesis.

Samples Reduced by Dithionite

8K : wide scan

In the control, all the expected [2Fe-2S] and [4Fe-4S] EPR signals can be observed, namely from centres N1, N2, N3, N4 of complex I, and possibly centre S1 of complex II (figure 3.17). Although, in theory, dithionite is a relatively strong reducing agent with a standard reduction potential (E'_0) of approximately -0.53V, in practice it reduces only complex I and complex II partially. Neither 25mM indomethacin nor 50mM aspirin exhibited any affect on these centres. However, with 100mM naproxen, a free radical signal appeared at g=2.0), and the intensity of the Fe-S signals was less when compared to the control, with a corresponding increase in the g=4.3 signal characteristic of free Fe^{3+}.

8K : narrow scan

This spectrum focuses on the Fe-S centres of the respiratory chain (figure 3.18). At the higher resolution, an additional small free radical is observed with the control. Indomethacin and aspirin were without effect on the EPR signals from the [2Fe-2S] and [4Fe-4S] clusters. Naproxen, on the other hand, seemed to produce a more prominent free radical signal and less intense Fe-S signals.

18K

At this temperature, it is apparent that in the naproxen-treated sample there is a significant decrease in the signal intensity of the N3 and N4 complex I centres, plus an emergence of a free radical (figure 3.19). This would suggest that naproxen prevents electron transport from complex I, since it prevented the full reduction of centre N3 by dithionite. Indomethacin and aspirin, however, were without significant effect.

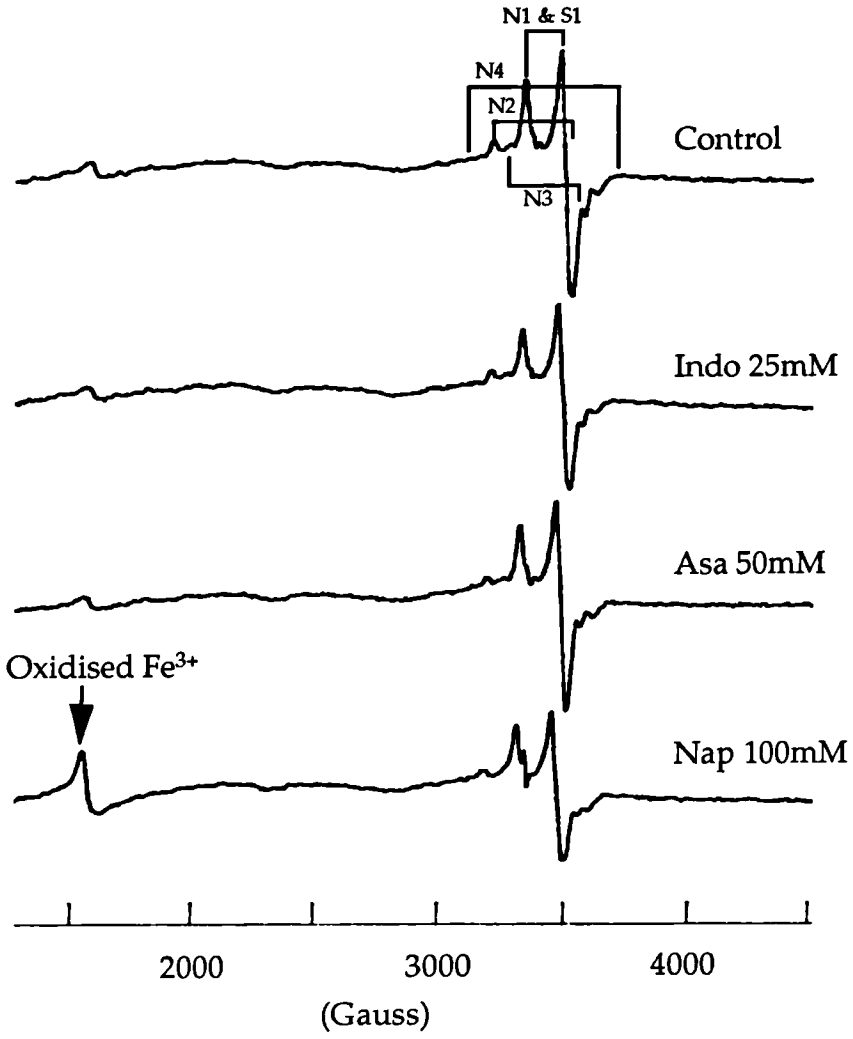


Figure 3.17 Wide scan EPR trace of dithionite-reduced BHM at 8K
EPR was carried out at 8K on BHM reduced by 4mM dithionite which was either untreated (control) or incubated with indomethacin (indo), aspirin (asa), or naproxen (nap), at the concentrations indicated. See main text for experimental details and abbreviations. This trace represents an average of 4 scans performed on a single BHM sample.

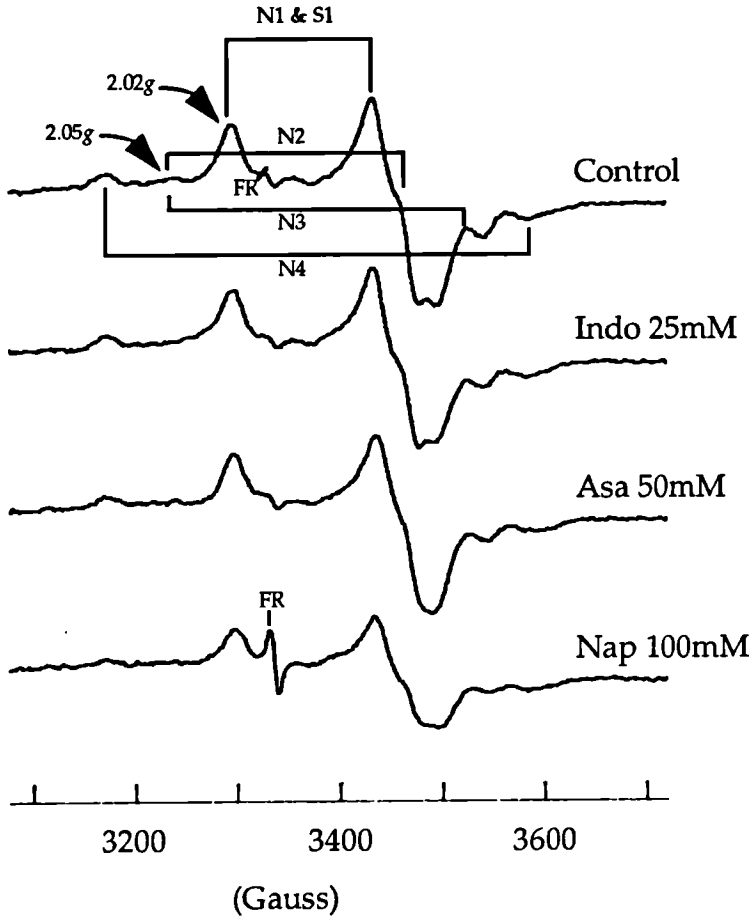


Figure 3.18 EPR trace of dithionite-reduced BHM at 8K

EPR was carried out at 8K on beef heart submitochondrial particles reduced by 4mM dithionite which were either untreated (control) or incubated with indomethacin (indo), aspirin (asa), or naproxen (nap), at the concentrations indicated. See main text for experimental details and other abbreviations. *g* = spectroscopic splitting factor; FR = free radical. This trace represents an average of 4 scans performed on a single BHM sample.

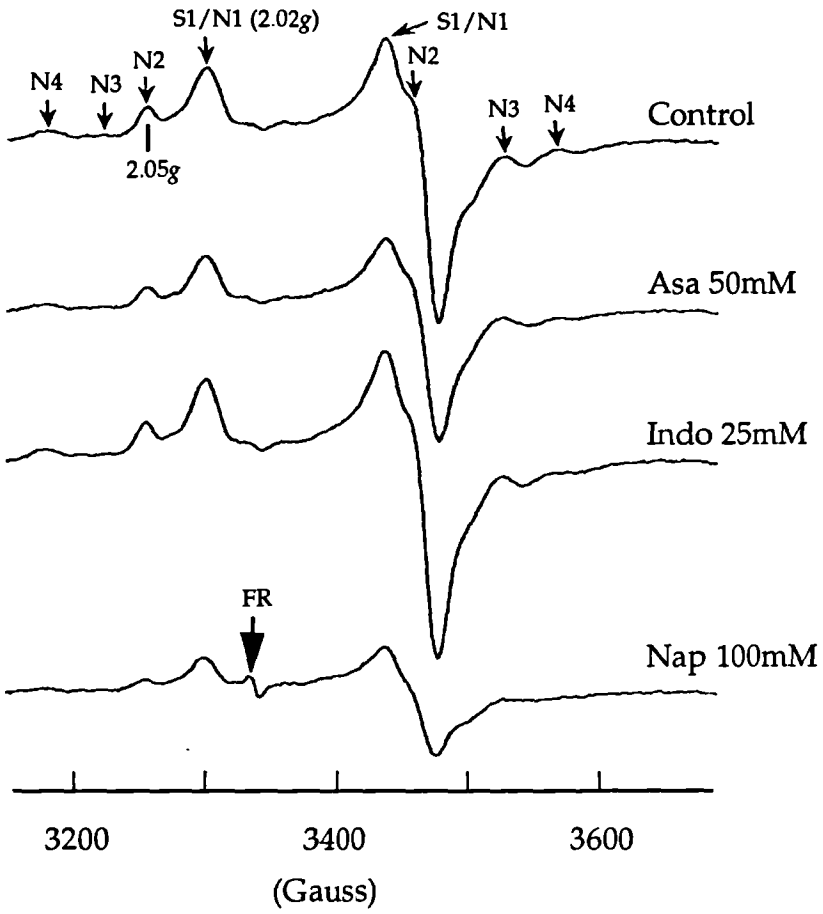


Figure 3.19 EPR trace of dithionite-reduced BHM at 18K

EPR was carried out at 18K on BHM reduced by 4mM dithionite which were either untreated (control) or incubated with indomethacin (indo), aspirin (asa), or naproxen (nap), at the concentrations indicated. See main text for experimental details and other abbreviations. *g* = spectroscopic splitting factor; FR = free radical. This trace represents an average of 4 scans performed on a single BHM sample.

Samples Reduced by 4mM NADH

8K

Signals corresponding to centres N1, N2, N3 and N4 are beginning to appear since NADH donates electrons specifically to complex I (figure 3.20). The prominent signal of S3 is also still apparent since it is not reduced by NADH, therefore remaining oxidised and EPR-active. Specific reduction of complex I suggests the Fe-S clusters of complex I seem to be greatly affected by 25mM indomethacin, less so by 100mM naproxen and not at all by 50mM aspirin, in stark contrast to the dithionite spectrum.

18K

By increasing the temperature slightly, centres N1 to N4 can be seen more clearly as can the effects of the drugs (figure 3.21). Signals from all centres, in particular centres 3 and 4, were affected by indomethacin and to a lesser extent by naproxen, but not by aspirin.

40K

At this temperature, signals from Cu_A^{2+} of cytochrome oxidase, centre N1, and the Rieske centre are all observed (figure 3.22). Cytochrome oxidase copper can be seen as it remains oxidised and EPR-active in the presence of NADH. In the control, however, there is an additional free radical signal which was also present in duplicate experiments; quite typical of mitochondrial preparations. With indomethacin and naproxen, there was no Rieske signal, suggesting that these drugs may block forward electron transport between complex I and complex III. However, aspirin has no effect on any of the signals. This would suggest that naproxen and indomethacin act at complex I whereas aspirin does not.

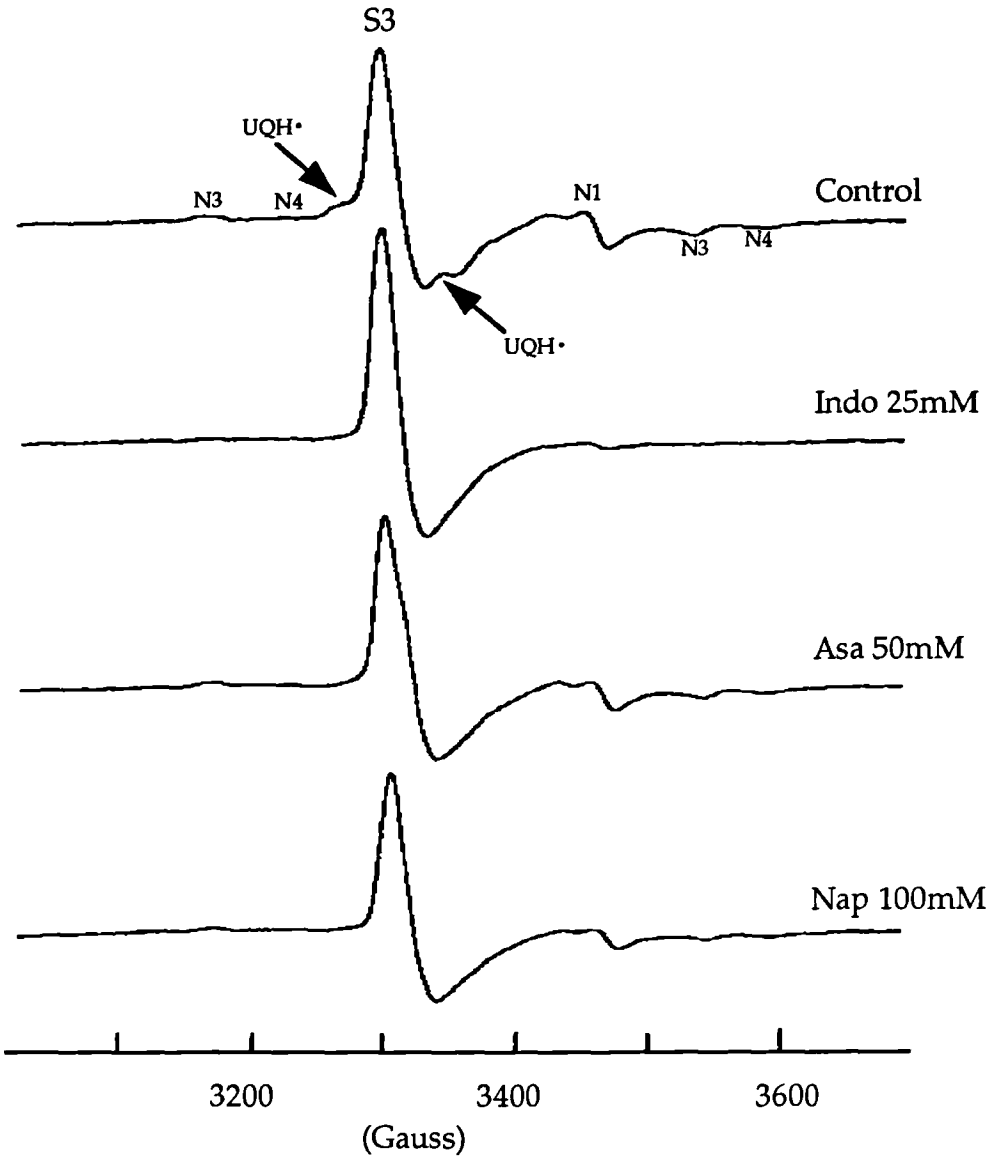


Figure 3.20 EPR trace of NADH-reduced BHM at 8K

EPR was performed at 8K on BHM reduced with 4mM NADH which were either untreated (control) or incubated with indomethacin (indo), aspirin (asa), or naproxen (nap), at the concentrationsindicated. See main text for experimental details and other abbreviations. UQH$^{\bullet}$ = interaction of centre S3 with semiquinone. This trace represents an average of 4 scans performed on a single BHM sample.

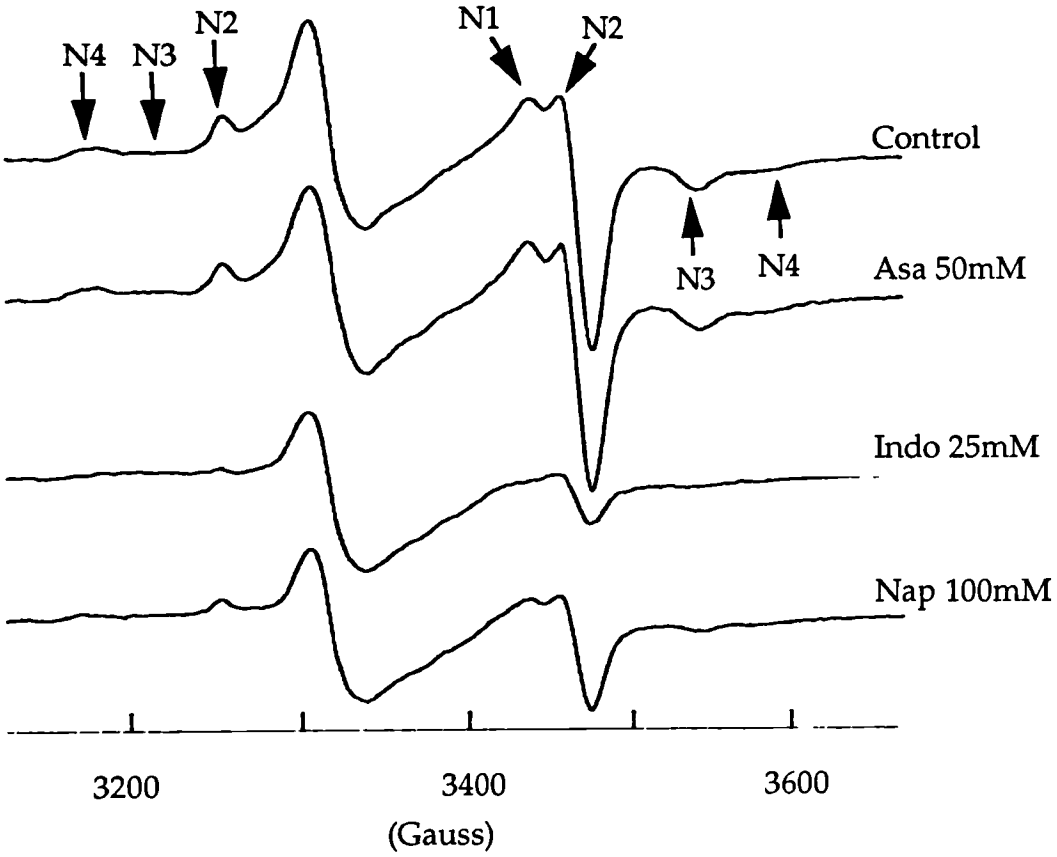


Figure 3.21 **EPR trace of NADH-reduced BHM at 18K**

EPR was carried out at 18K on beef heart submitochondrial particles reduced by 4mM NADH which were either untreated (control), or incubated with indomethacin (indo), aspirin (asa), or naproxen (nap), at the concentrations shown. See main text for experimental details and abbreviations. This trace represents an average of 4 scans performed on a single BHM sample.

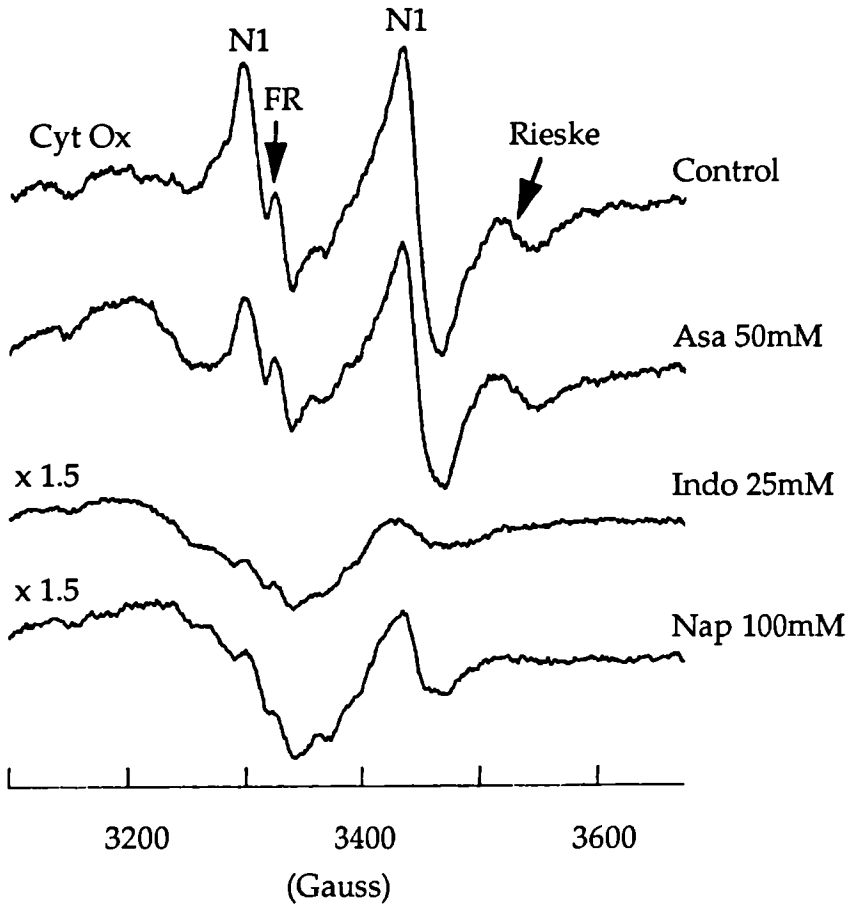


Figure 3.22 EPR trace of NADH-reduced BHM at 40K

EPR was carried out at 18K on BHM reduced by 4mM NADH which were either untreated (control), or treated with indomethacin (indo), aspirin (asa), or naproxen (nap), at the concentrations shown. See main text for experimental details and other abbreviations. FR = free radical; Cyt Ox = cytochrome oxidase. This trace represents an average of 4 scans performed on a single BHM sample.

Samples Reduced by 60mM Succinate

Controls at 8, 18 and 40K

Control samples were run at 8, 18 and 40K. The clearest signals were seen at 40K (figure 3.23). Therefore, the drug effect was not tested at the lower temperatures following succinate reduction.

40K

Succinate reduces complex II and can sometimes also reduce complex III due to forward electron transfer by the respiratory chain. Signals from centre S1 and the Rieske centre are observed. A signal corresponding to the Cu_A^{2+} of cytochrome oxidase is also apparent since it remains oxidised, thus EPR-detectable, in the presence of succinate (figure 3.24). A free radical signal could also be seen. With aspirin and indomethacin the S1 signal intensity is decreased. With naproxen, however, this S1 signal is not present at all and there appears to be a very intense NO signal instead.

Table 3.3 summarises the effects of the NSAIDs on the various Fe-S centres. Overall, these studies showed an interaction of the NSAIDs with Fe-S clusters of complex I, II and III. No one complex in particular was targeted by the drugs, in agreement with the oxygen electrode studies. Also, more than once, a free radical signal appeared, in accordance with the idea that reactive oxygen species may play a part in NSAID-induced gastrointestinal damage. Similarly, naproxen appeared to evoke the release of an NO group, a finding also reported with indomethacin *in vivo*.

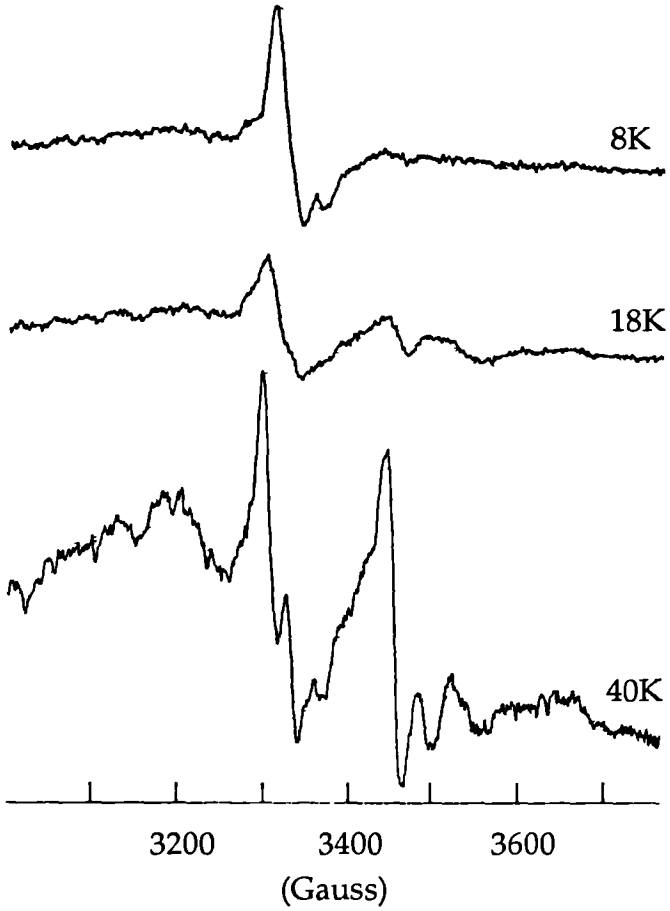


Figure 3.23 EPR trace of succinate-reduced BHM

EPR was carried out at 8, 18 and 40K on beef heart submitochondrial particles reduced with 60mM succinate. See main text for experimental details. This trace represents an average of 4 scans performed on a single BHM sample.

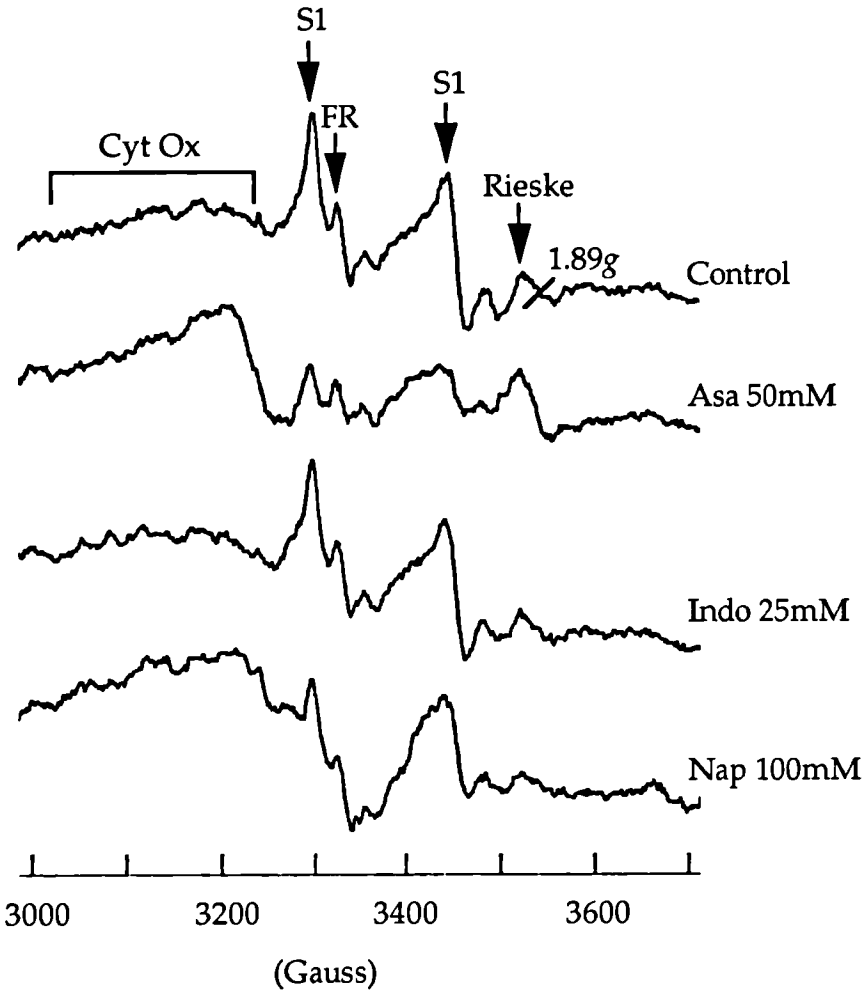


Figure 3.24 **EPR trace of succinate-reduced BHM at 40K**

EPR was carried out on BHM at 40K reduced by 60mM succinate which were either untreated (control) or incubated with aspirin (asa), indomethacin (indo), or naproxen (nap), at the concentrations indicated. See main text for experimental details. S1 = [2Fe-2S] cluster of complex II; Cyt Ox = cytochrome oxidase; FR = free radical; *g* = spectroscopic splitting factor. This trace represents an average of 4 scans performed on a single BHM sample.

Table 3.3 Effect of NSAIDs on the Fe-S clusters of complexes I, II and III

Experimental Conditions	Fe-S Clusters Targeted	Conclusions
Oxidised	Centre S3	Interference with the interaction of S3 with ubiquinone: electron transport block at complex II. Also NO signal seen in the presence of naproxen
Dithionite-reduced	All [2Fe-2S] and [4Fe-4S] clusters	Only naproxen caused a reduction in the signals of N3 & N4, and gave a free radical: block at complex I
NADH-reduced	Complex I	Indomethacin and naproxen reduced signals of complex I centres and abolished Rieske signal: block between complex I and complex III
Succinate-reduced	Complex II	Naproxen abolishes, and indomethacin and aspirin reduces, S1 signal: block at complex II. Naproxen also gives NO signal

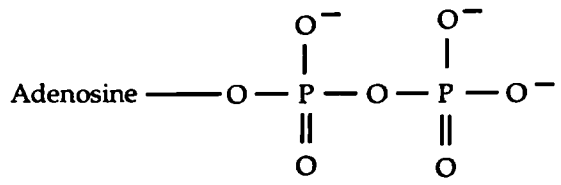
CHAPTER FOUR

DECREASE IN MITOCHONDRIAL ATP PRODUCTION BY NSAIDS

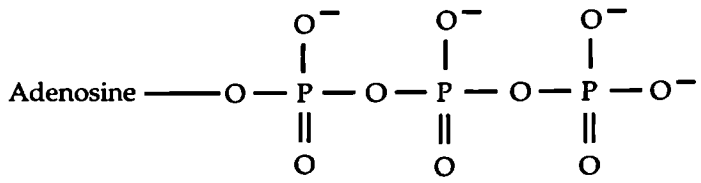
ATP (figure 4.1) is a compound with a relatively high free-energy of hydrolysis used by a large number of cellular pathways. The process of oxidative phosphorylation is responsible for the synthesis of at least 90% of the total ATP formed in most aerobic cells containing mitochondria. The remaining ATP is generated during glycolysis and the citric acid cycle via GTP (Mayes, 1993). For the majority of ATP synthesis, the phosphate required for ADP phosphorylation is imported into the mitochondria by a phosphate carrier, and the ATP generated is exported to the cytosol in exchange for ADP by the adenine nucleotide carrier (ANC) (figure 4.2). The turnover of ATP is very high, being continuously regenerated from ADP. This is because it acts as an immediate donor, rather than long-term storage form, of free energy. In the cell, an ATP molecule is consumed within a minute following its formation (Stryer, 1988).

Depletion of ATP is caused by many compounds, resulting in a variety of biochemical changes. Perhaps the most common mechanism of ATP depletion in the cell is by interference with oxidative phosphorylation, inhibition of electron transport, or depletion of NADH. Excessive ATP utilisation or sequestration, or DNA damage which causes the activation of poly(ADP-ribose)polymerase, are other mechanisms which may reduce ATP levels.

A lack of ATP in the cell would mean that active transport, into, out of and within the cell, is compromised or arrested, resulting in changes in Na^+, K^+ and Ca^{2+} ion concentrations in particular compartments. In addition, there may be a decrease in certain synthetic biochemical processes such as protein synthesis, gluconeogenesis and lipid synthesis. At the tissue level, this could mean that hepatocytes, for example, will not produce bile efficiently or that



Adenosine Diphosphate (ADP)



Adenosine Triphosphate (ATP)

Figure 4.1 The structures of ADP and ATP

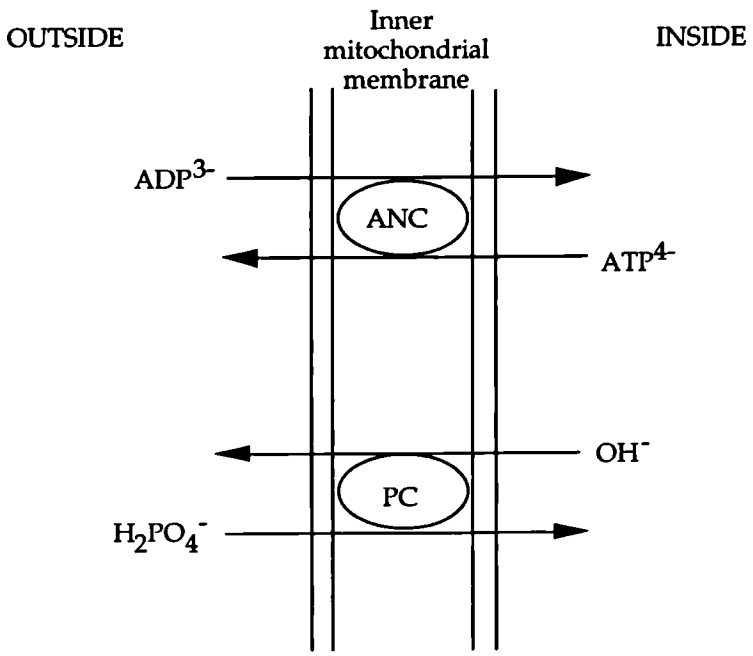


Figure 4.2 Carrier systems in the mitochondrial membrane

ANC = adenine nucleotide carrier; PC = phosphate carrier; ADP^{3-} = adenosine diphosphate; ATP^{4-} = adenosine triphosphate; $H_2PO_4^-$ = phosphate; and OH^- = hydroxyl. Adapted from Mayes (1993).

proximal tubules will not actively reabsorb essential amino acids and glucose (Timbrell, 1991).

As shown by the studies of chapters 2 and 3, NSAIDs uncouple oxidative phosphorylation and inhibit mitochondrial electron transport. Both of these effects would result in an inhibition of ATP synthesis. This would entail a series of events, which could ultimately play a part in the gastrointestinal damage seen with these drugs. The aim of the present section is to directly measure the effect of NSAIDs on mitochondrial ATP content.

4.1 Materials and Methods

For measuring oxygen consumption, the materials and methods were the same as before (chapter 2, section 2.4). For measuring ATP levels:

4.1.1 Materials

β-mercaptoethanol, potassium hydroxide (KOH), nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate dehydrogenase (G-6-PD) and hexokinase were supplied by the Sigma Chemical Company Ltd., Dorset, England. Perchloric acid 70% (PCA) and D-glucose were obtained from BDH Ltd., Poole, England. HEPES was supplied by Fluka Chemicals, Dorset, England.

4.1.2 Methods

Mitochondria from rat liver were isolated as before. The P/O and respiratory control ratios of the mitochondria were measured to ensure they were tightly coupled.

On adding 0.67mM ADP to 2.1 to 8.9 mg mitochondria respiring on 1mM succinate, it took approximately 5 minutes for all the ADP to be converted to ATP i.e. for state 3 to go back to state 4 respiration. Therefore, 1 minute after addition of ADP there should still be sufficient ATP synthesis taking place.

The effect of increasing concentrations of the NSAIDs indomethacin, aspirin and naproxen, the analgesic paracetamol, and the uncoupler FCCP, were tested on ATP levels. The concentrations of the drugs were approximately in the same range as that used previously for oxygen uptake studies. The concentrations of FCCP used were those shown previously to strongly stimulate oxygen uptake. Also tested were the respiratory inhibitors antimycin A (0.01mM) and cyanide (1mM). Again, these concentrations of the inhibitors were based on those shown previously to strongly inhibit oxygen consumption.

PCA Extraction

To measure the effects of compounds on ATP synthesis, the following were incubated for 1 minute at 30°C: oxygen electrode buffer, coupled mitochondria (2.1 to 8.9 mg), 1mM succinate, 0.67mM ADP, and increasing concentrations of the drug or solvent. The reaction was stopped by the addition of ice-cold PCA (final concentration of 20%) which precipitates proteins, leaving cytosolic elements in the supernatant. This cloudy white suspension was spun in a

microcentrifuge (MSE; Medical and Scientific Equipment Ltd) for 5 minutes and the supernatant transferred to fresh vials.

1.5M KOH was added to this supernatant, producing a cloudy mixture, and spun again for 5 minutes in the microcentrifuge to precipitate and remove potassium perchlorate. The supernatant was then neutralised by adding solid HEPES, and stored at -70°C until assayed.

ATP Assay

An enzymatic assay was used to assess ATP levels.

Principle

ATP phosphorylates glucose via hexokinase:

$$\text{Glucose} + \text{ATP} \xrightarrow[Mg^{2+}]{\textit{hexokinase}} \text{Glucose-6-Phosphate} + \text{ADP}$$

The glucose-6-phosphate (glucose-6-P) is then converted to glucose-6-phosphate dehydrogenase (G-6-PD):

$$\text{Glucose-6-P} + \text{NADP}^{+} \xrightarrow{\textit{G-6-PD}} \text{6-PGA} + \text{NADPH} + \text{H}^{+}$$

The change in absorbance at 340nm, resulting from the reduction of NADP to NADPH, can be measured. In the presence of excess glucose, this change in absorbance is proportional to the amount of ATP in the mixture (Beutler, 1975).

Experimental Procedure

The PCA extracts were thawed and kept on ice. The following solutions were then added to a 2ml cuvette at the volumes shown below:

Solution	Volume (µl)
Tris-HCl 1M, EDTA 5mM, pH8	100
$MgCl_2$ 0.1M	20
NADP 2mM	200
Glucose 20mM	50
Distilled water	414
G-6-PD 50 units/ml	6

(Note, both G-6-PD and hexokinase were made up in a stabilising solution comprising of 20% β-mercaptoethanol in Na_2EDTA:Na_4EDTA (1:1)).

The cuvettes were warmed on a water bath to 37°C, then 200µl of the PCA extract was added. This was mixed and the absorbance at 340nm was read using a double beam spectrophotometer (Cecil Instruments) to give a baseline reading. 10µl hexokinase (400units/ml) was then added, mixed, incubated for 30 minutes at 37°C, and the absorbance again measured.

Therefore,

$$\text{ATP concentration} = \frac{\Delta OD \times \text{overall dilution factor} \times 5 \times 1000}{6.22}$$

$\Delta OD = (OD_{+hex})-(OD_{baseline})$

200ul PCA added to 1ml in cuvette = 1/5 dilution

mmoles

extinction coefficient (mM) for NADH

overall dilution factor = final volume/ original volume

original vol (µl)=buffer+succinate+ADP+drug/solvent+mitochondria

final vol (µl)=original vol+PCA+KOH

4.2 Results

4.2.1 Effect of NSAIDs on ATP Production

Figure 4.3a shows how all three NSAIDs tested, with increasing concentrations (0 to 17mM), were associated with a decrease in ATP production. The drug giving the greatest reduction in ATP production with the lowest concentration was indomethacin > naproxen > aspirin. Paracetamol did not reduce ATP production. FCCP (0 to 10μM) decreased ATP production to a maximum of 66% of control (figure 4.3b).

4.2.2 Effect of Respiratory Inhibitors on ATP Production

Figure 4.4 shows the effect of the antimycin A (0.01mM) and cyanide (1mM) on ATP formation. Antimycin A only inhibited to 88% of control and cyanide to 78% of control.

4.3 Discussion

Although the uncoupling of oxidative phosphorylation implies inhibition of ATP formation, a direct measure of ATP production is necessary in order to definitely state ATP production is inhibited by NSAID action. The present experiments show that all the NSAIDs studied decreased ATP production. This is logical since oxidative phosphorylation is responsible for ATP generation. Indomethacin was again the most potent at inhibiting ATP production as it was at uncoupling oxidative phosphorylation and inhibiting electron transport (as shown by the oxygen electrode studies).

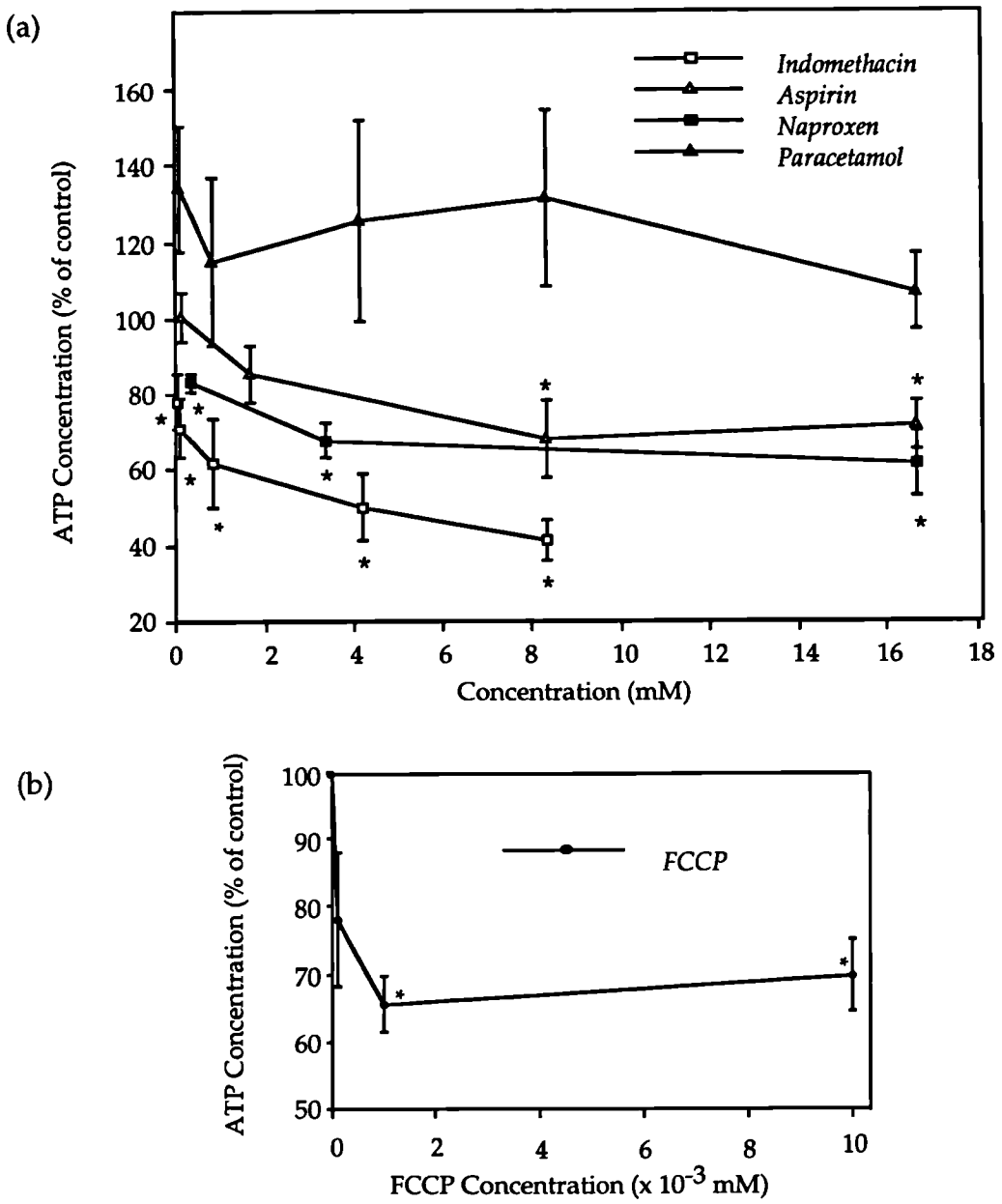


Figure 4.3 Effect of drugs on mitochondrial ATP production

To mitochondria (2.1-8.9 mg) was added 1mM succinate, 0.67mM ADP, then (a) NSAIDs / paracetamol or (b) FCCP. After 1 minute incubation at 30°C, the reaction was stopped by the addition of PCA. Neutralisation with KOH/HEPES, followed by addition to a reaction mixture (see main text), enabled measurement of ATP production by the samples by reading absorbance at 340nm. Results are mean of 3 experiments ± SEM (3 separate liver preparations). * Significantly different from control, $P < 0.05$, Student's t-test.

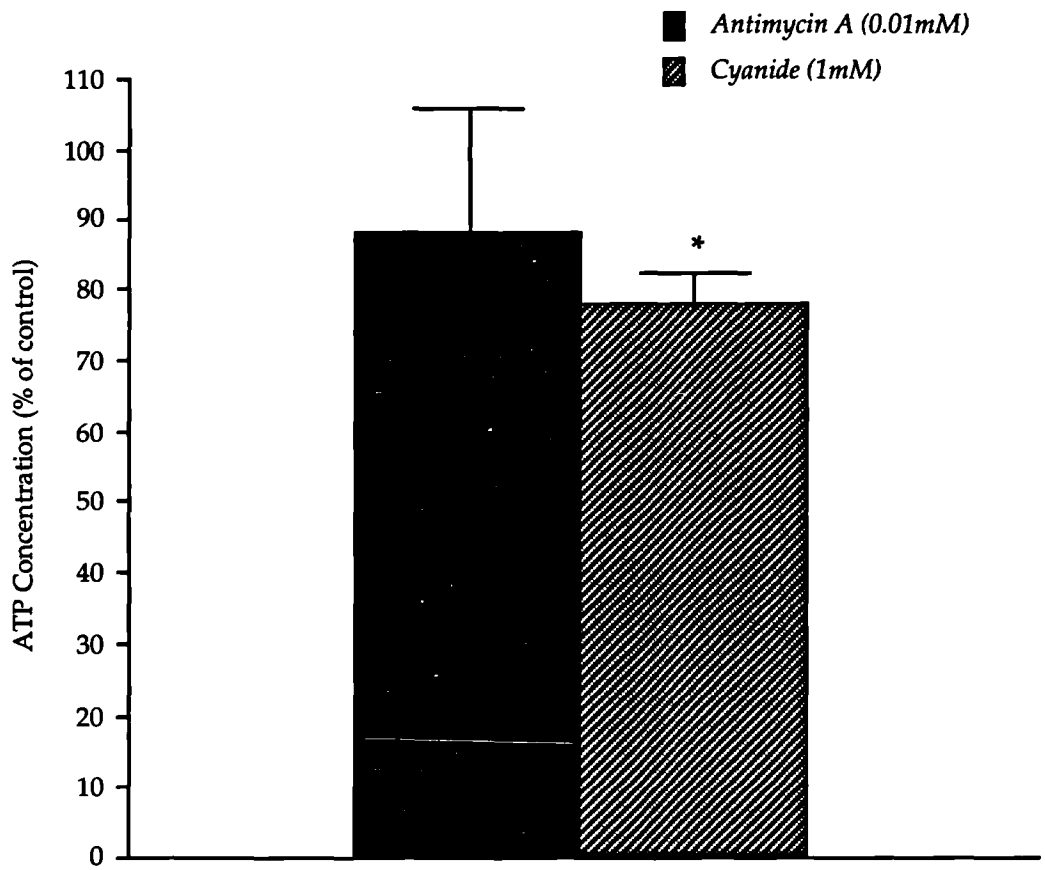


Figure 4.4 Effect of respiratory inhibitors on mitochondrial ATP production
To mitochondria (2.1-8.9 mg) was added 1mM succinate, 0.67mM ADP, then antimycin A (0.01mM)/ cyanide (1mM)/ solvent. After 1 minute incubation at 30°C, the reaction was stopped by the addition of PCA. Neutralisation with KOH/HEPES, then addition to a reaction mixture (see main text), enabled measurement of ATP production by the samples by reading absorbance at 340nm. Each result represents the mean of 3 experiments ± SEM (3 separate liver preparations). * Significantly different from control, $P < 0.05$, Student's t-test.

The fact that paracetamol did not reduce ATP formation at concentrations previously shown to inhibit electron transport by approximately 50% (see chapter 3) was surprising and revealing. Though this drug does not uncouple oxidative phosphorylation, it does inhibit respiration as shown by a decrease in mitochondrial oxygen consumption, which would also decrease ATP production. It would appear that sufficient activity remains in the remaining uninhibited electron transfer components to maintain ATP production over the time period of these measurements. The conclusion from these studies, therefore, is that it is the uncoupling action of the NSAIDs which is primarily responsible for a decline in ATP generation in mitochondria, rather than an inhibitory action.

ATP measurement as an indicator of uncoupling effect has previously been reported with these drugs. As with the present study, others have measured ATP levels as an indicator of ATP synthesis. For example, the mucosal ATP content of the human jejunum (Arvanitakis *et al.*, 1977) and also in bullfrog stomach (Spenney and Bhown, 1977) is decreased by exposure to aspirin. A depletion in ATP production has also been observed in human neutrophils by the NSAIDs indomethacin, piroxicam and sodium salicylate (Cronstein *et al.*, 1994), and in human lymphocytes, used as a model for testing drug effect on human mitochondria, by the drug ibuprofen (Fromenty *et al.*, 1993).

A stimulation of ATPase activity by NSAIDs, which causes a decrease in ATP synthesis, has also been well documented (Byczkowski and Korolkiewicz, 1977; Tokumitsu *et al.*, 1977; Baños and Reyes, 1989; and Salguiero-Pagadigorria *et al.*, 1996). Contradictory findings, however, have been demonstrated by Chatterjee and Stefanovich (1976), where indomethacin (IC_{50}

0.55mM) and aspirin (IC_{50} 13mM) appeared to inhibit rat liver mitochondrial ATPase, the reason for this inconsistency is unclear.

Mingatto *et al.* (1996) measured actual ATP synthesis and found, in accordance with this study, that aspirin and other NSAIDs piroxicam, diclofenac, dipyrone, mefenamic acid, inhibited the rate of ATP synthesis. Unlike the present study however, paracetamol also inhibited ATP synthesis, though requiring higher concentrations. A drop in ATP generation by paracetamol was similarly demonstrated by Porter and Dawson (1979) in kidney cells. In the study of Mingatto *et al.* (1996) the drugs were incubated for 10 minutes with the mitochondria before measuring ATP synthesis, and with the study of Porter and Dawson (1979) the incubation period was 1 hour. This would suggest that, had paracetamol been incubated for longer than 1 minute in the present study, it too may have brought about a decrease in ATP production.

The importance of incubation time is highlighted in the present study by the FCCP results and those of the inhibitors antimycin A and cyanide. FCCP, being a highly potent uncoupler of oxidative phosphorylation, and antimycin A and cyanide being strong inhibitors, would be expected to cause a drastic decrease in mitochondrial ATP synthesis. However, these compounds gave no more than a 35% drop in ATP production. It would suggest that 1 minute incubation with the compounds was not sufficient to fully deplete ATP production. Alternatively, higher concentrations may be required, since in another study it was found that 37μM FCCP caused an 80% reduction in spontaneous ATP production (Nässberger, 1990).

Nässberger (1990) also found that the solvent DMSO caused an increase in ATP output by about 20%, though the mechanism by which this occurred was not investigated. Despite the final concentration of DMSO never exceeded 2°o

in my experiments and drug effect being calculated as a percentage of solvent effect, the ATP-promoting effect of DMSO may explain why ATP production never reached zero.

4.3.1 Significance of Decreased ATP Production

It can be envisaged, therefore, that a lack of ATP generation caused by the action of NSAIDs in uncoupling oxidative phosphorylation or possibly even by inhibition of dehydrogenase enzymes in the tricarboxylic acid cycle (Smith and Dawkins, 1971), could play a part in some of the gastrointestinal side-effects seen with these drugs. Reduced ATP production would cause calcium ions (Ca^{2+}) to leak out of the mitochondria (Carafoli, 1987). Increased cytosolic Ca^{2+} may lead to activation of Ca^{2+}-sensitive enzymes such as proteases, endonucleases and phospholipases (Tuena de Gomes-Puyou *et al.*, 1980). Phospholipase A_2 activation would result in the release of reactive oxygen species from membrane phospholipids (Jain and Shohet, 1981). Protease activation could evoke microtubule depolarisation, affecting the cytoskeleton (Tada *et al.*, 1974), which could in turn be responsible for the increase in intestinal permeability seen with NSAIDs (Somasundaram *et al.*, 1995).

Another consequence of reduced ATP production, would be a compensatory stimulation of glycolysis (see chapter 2, section 2.6). However, enterocytes have low phosphofructokinase (PFK), and high glucose-6-phosphate (G-6-P) dehydrogenase and phosphogluconate dehydrogenase, activities, and may not be able to increase their glucose consumption as much as other cells by glycolysis.

A possible consequence of stimulated glycolysis in enterocytes, is that the pyruvate formed in glycolysis is converted into lactate instead of acetyl CoA

(figure 4.5), due to the low activity of pyruvate dehydrogenase in these cells (Jongab *et al.*, 1992). This would result in intracellular acidosis. If there was a prevalent reduction in blood flow, cell viability would be further compromised.

By inhibiting ATP generation, the NSAIDs would deprive the organism of the numerous other ATP-dependent reactions. For example, an inflammatory stimulus initiates many energy-dependent reactions such as vasodilatation, histamine release, movement of leukocytes into the inflamed area. This requires a continuous supply of ATP to sustain the inflammatory response (Whitehouse, 1968). This is one explanation of why, in inflamed areas of tissue, ATP content is higher since ATP synthesis is occurring more rapidly, than the adjacent "normal" tissue (Kalbhen, 1963). Decreasing ATP synthesis would significantly affect the rate at which the whole inflammatory response, including the ultimate reparative processes, will occur (Whitehouse, 1968).

A distinct correlation between decreased ATP production and NSAID-gastrointestinal damage has been demonstrated. For example, decreased ATP production has been measured in the areas of the gastric mucosa most prone to ulceration by aspirin ingestion, the corpus and pyloric gland regions (Jörgensen *et al.*, 1976b). Also, the appearance of gastric mucosal damage induced by aspirin has been associated with an increase in ATP breakdown to ADP. In indomethacin-induced damage, the changes in tissue ATP metabolism have actually been shown to precede the development of macroscopic gastric mucosal injury (Mózsik *et al.*, 1992).

Paradoxically, the decrease of ATP synthesis by antiinflammatory drugs, was once even thought to be beneficial (Whitehouse, 1968). In chronic

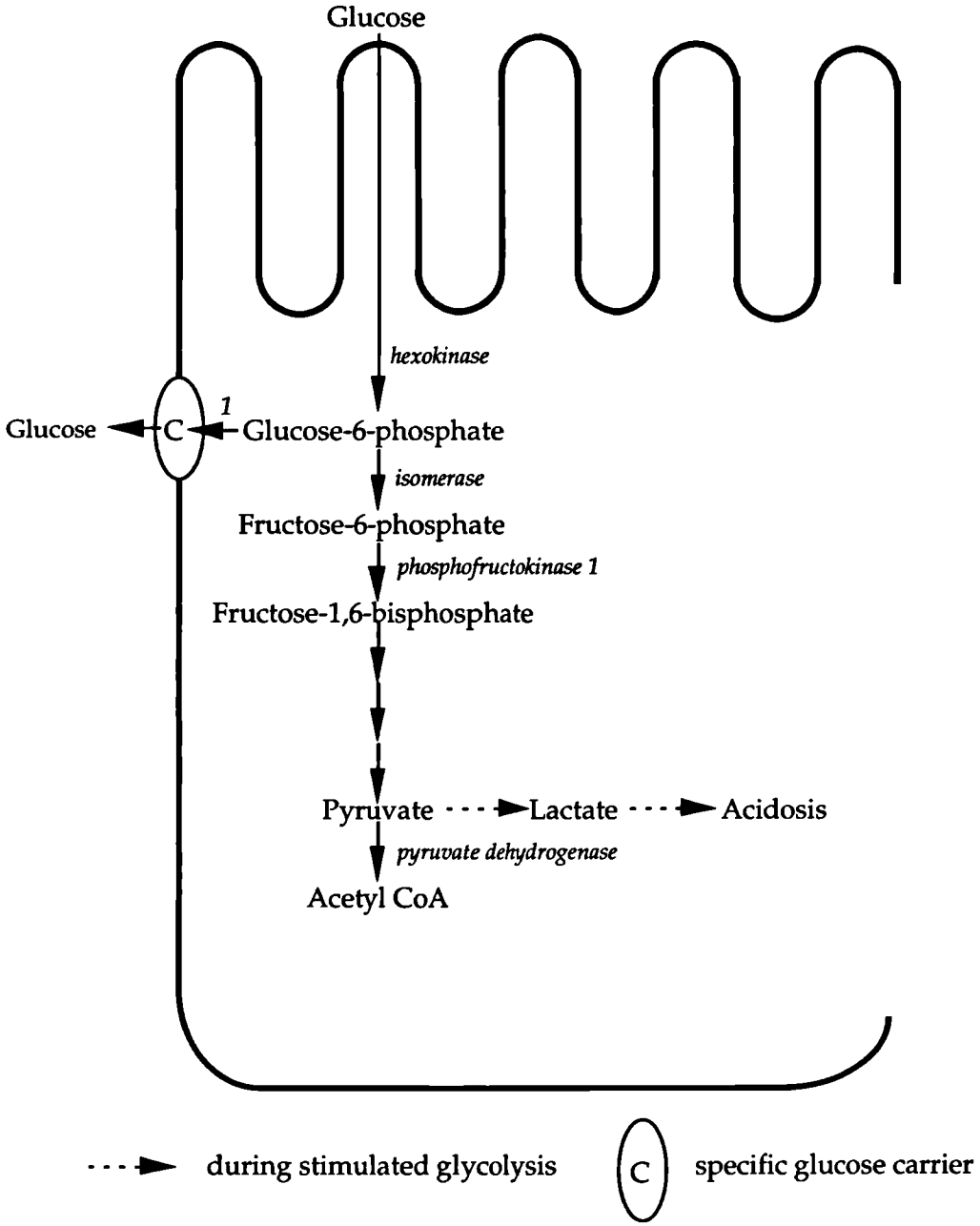


Figure 4.5 Fate of glucose in enterocytes

In the enterocyte most of the glucose-6-phosphate is metabolised by the hexose monophosphate (HMP) pathway or is transported out by specific carriers (C) at the basolateral borders. If glycolysis is stimulated, then lactate is formed. *1 = glucose-6-phosphatase.* Adapted from Somasundaram *et al.* (1995).

inflammatory disease, connective tissue proliferation and polymer biosynthesis involved in the pathogenesis of joint stiffness, are inhibited by these drugs presumed to be because they diminish the energy supply supporting these reactions in these tissues.

But it must be emphasised that decreased ATP generation alone is not in itself a biochemical indication of gastrointestinal damage. For example, when mucosal damage is induced by pylorus ligation, stress, ethanol, or reserpine, there is a significant decrease of tissue ATP at the early stage of ulcer development, but its level is later elevated (Mózsik and Jávor, 1988; and Mózsik *et al.*, 1990). This would tie in with the suggestion that mitochondrial disturbances together with inhibition of prostaglandin synthesis are both involved at the initial stage of NSAID enteropathy (Somasundaram *et al.*, 1992).

CHAPTER FIVE

EFFECT OF NSAIDS ON INTESTINAL CELL MONOLAYERS

Small intestinal injury caused by NSAIDs is more common than those in the stomach or duodenum, affecting 60-70% of patients taking these drugs for more than six months (Bjarnason *et al.*, 1993). Inflammation in the small intestine caused by NSAIDs may be complicated by mild bleeding and protein loss, which may lead to iron deficiency anaemia and hypoalbuminaemia (Bjarnason *et al.*, 1987b). Diaphragmatic-like strictures may also occasionally affect patients, which could require surgery, though relatively uncommon (Bjarnason *et al.*, 1988). In the stomach or duodenum, on the other hand, the pattern of NSAID-induced damage is different and often more dramatic, whereby ulcers may perforate and bleed.

Numerous factors have been implicated in the pathogenesis of NSAID enteropathy, such as impaired epithelial barrier function (Somasundaram *et al.*, 1995), bile, luminal bacteria (Yamada *et al.*, 1993), and enterohepatic recirculation of the NSAID (Wax *et al.*, 1970). The relative importance of these factors in the pathogenesis, has still not been determined (Reuter *et al.*, 1997). In addition, inhibition of prostaglandin synthesis seems not to be of major importance in the pathogenesis of small intestinal injury caused by these drugs (Whittle, 1981; Davies *et al.*, 1993; Rooney and Jenkins, 1990; and Somasundaram *et al.*, 1995).

Nevertheless, it is suggested that the change in intestinal permeability is the central mechanism in the whole framework of NSAID-induced enteropathy (Somasundaram *et al.*, 1995). The increase in intestinal permeability caused by NSAIDs, enables the biochemical damage (e.g. the mitochondrial changes), to be converted into the macroscopic damage, by facilitating mucosal exposure to luminal aggressive factors thereby eliciting neutrophil chemotaxis.

5.1 Structure and Function of Tight Junctions

The intestinal wall is lined by a layer of epithelial cells. The apical membrane of this polarised monolayer is in contact with the luminal space of the intestine, while the basolateral (or serosal) membrane is in contact with the body fluid which provides nutrients for the cell (Simons and Fuller, 1985). The function of the normal healthy gastrointestinal epithelium is to permit the absorption of nutrients, electrolytes, and water, whilst at the same time restricting the passage of larger potentially toxic compounds from the lumen into the systemic circulation (Salzman *et al.*, 1995). This "selective permeability" of the intestinal mucosa is mediated by intercellular tight junctions ("zonula occludens") (Madara, 1989).

The tight junction is a narrow belt-like structure, measuring between 0.1 and 0.7 μm in width, on the plasma membrane of transporting epithelial cells (Madara *et al.*, 1988a; and Schneeberger and Lynch, 1992). The junction generally resides near the apex of the cell, forming a continuous circumferential seal between adjacent cells, thereby selectively restricting the permeation of ions and nonelectrolytes, according to their charge and size, between the lumen and the basolateral space.

There are only two pathways by which molecules can cross the epithelium: the paracellular or the transcellular route (figure 5.1). At least 85% of passive ion flow across the mammalian small intestine takes the paracellular route (Frizzell and Shultz, 1972). Tight junctions restrict flow through the paracellular pathway. Tight junctional permeability is thought to be under cytoskeletal control, since an increase in paracellular permeability to small molecules may involve contraction of the perijunctional actinomyosin ring

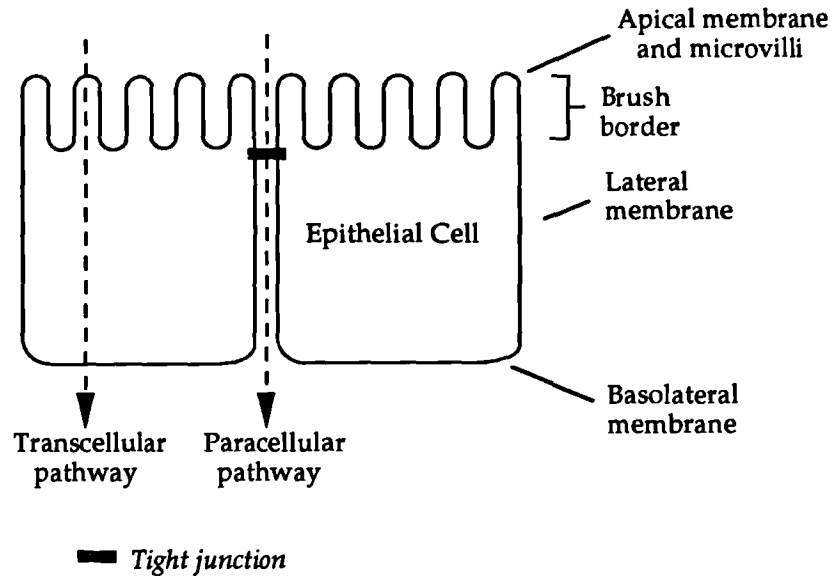


Figure 5.1 Passage of molecules through epithelial cells
The tight junctions reside between the cells, toward the apical side. Molecules can pass through the cells (transcellular) or in between the cells (paracellular). Modified from Madara (1988).

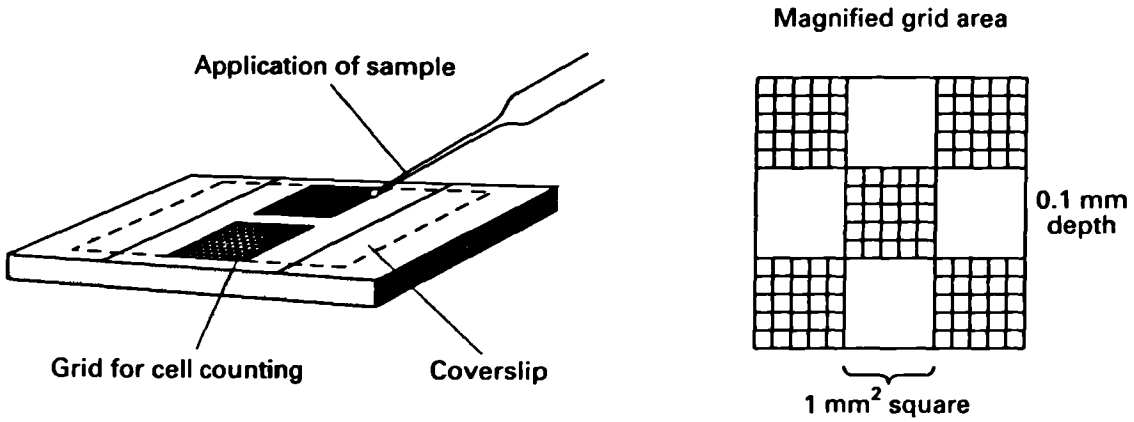


Figure 5.2 Cell counting chamber
The haemocytometer is a modified microscopic slide engraved with two identical grids of known area. Each of these grids comprise of 5 identical, smaller sets of grids $1mm^2$ square and 0.1mm depth. Therefore, when cell suspension is introduced by a Pasteur pipette and the coverslip correctly positioned, the volume of liquid per grid is $0.1mm^3$. If the total no. of viable cells in one of these grids are counted, then when n = no. of cells in $1mm^3$, the no. of cells in $1cm^3$ (i.e. 1ml) = $n \times 10^4$ and cells/cm^2 = $n \times 10^5$. Obtained from Butler (1996).

(Madara and Pappenheimer, 1987). Cytoskeletal components, in turn, appear to be partially regulated by ATP which elicits contraction (Burgess, 1982). Therefore, perturbed ATP formation (e.g. by uncoupling of oxidative phosphorylation) may bring about an increase in intestinal permeability, as hypothesised in this thesis.

5.2 Advantages of Using Cell Lines

Traditionally, investigations of intestinal permeability have used *in situ* or *in vivo* animal models which are not only expensive, but time-consuming, require large amounts of sample, and can show great variability due to luminal contents, mucus layer, hepatic clearance, and animal-to-animal or species-to-species differences (Cogburn *et al.*, 1991). *Ex vivo* transport models have also been used with everted intestinal sacs, mucosal tracts mounted in Ussing chambers (Schultz and Zalusky, 1964), luminally or vascularly perfused intestine (Hartmann and Plauth; and Schumann and Strugala, 1989), or brush border membrane vesicles (Kessler *et al.*, 1978). However, such techniques have proved to be laborious, difficult to reproduce, and limited by rapid tissue degradation following removal from the body. Moreover, although looking at effects on brush border membrane vesicles is simpler than using whole-tissue models and have a larger capacity, they can only be used to study the effects on the cell membranes (Anderberg *et al.*, 1992). No information about the possible effects on the permeability across tight junctions can be obtained from such systems.

Another major difficulty in measuring the effect of compounds on intestinal function is due to the complex geometric structure and cellular heterogeneity of this organ (Grasset *et al.*, 1984). As a result, access to the apical (luminal) or

basolateral (serosal) surfaces of the epithelium is impaired by the presence of subadjacent tissues. The use of cultured intestinal cells will overcome these difficulties, while at the same time maintaining polarised morphological and functional characteristics typical of their role *in vivo*. The availability of human cells, the low variability between replicates, the necessity for small sample and drug volumes, the ability to examine large numbers of samples, and clearer interpretation of results due to cellular homogeneity, are all additional advantages of using cultured cells to those techniques formerly used to measure intestinal permeability (Hilgers *et al.*, 1990; and Cogburn *et al.*, 1991).

The use of cells directly isolated from intestinal epithelium (primary cells) would be the ideal choice, and although some advances toward this goal has been made (Quaroni *et al.*, 1979), they are not yet available. This is because these cells are difficult to culture, have limited viability (~30 to 40 minutes), and there is a risk of contamination by microorganisms normally found in the gastrointestinal tract (Pinkus, 1975; Moyer, 1983; and Dharmsathaphorn and Madara, 1990). Moreover, during isolation of mucosal cells, a loss of polarity together with substantial damage of the cells may occur. The use of cell lines are preferred since their uniformity in culture eliminates the problems created by mixed cell types and different degrees of viability, which occurs when isolated intestinal cells are studied (Dharmsathaphorn *et al.*, 1984).

Human adenocarcinoma cell lines reproducibly exhibit a number of structural and functional properties characteristic of differentiated intestinal epithelial cells (Pinto *et al.*, 1983). Therefore, these cell lines provide an excellent means of studying intestinal function, such as paracellular permeability.

These cell lines can be seeded onto to permeable filter supports. Under appropriate culture conditions, the surface of the cells adherent to the filter forms the basolateral side, while the nonadherent surface forms the apical side. As *in vivo*, tight junctions can develop between cells grown on filters, and similarly are responsible for their impermeability and polarity (Cereijido *et al.*, 1989).

5.3 The Caco-2 Cell Line

The Caco-2 cell line is obtained from human colorectal adenocarcinoma (Fogh *et al.*, 1977). Despite its colonic origin, when cultured the Caco-2 cell line grows as a monolayer which, at confluency, undergoes spontaneous differentiation into polarised, columnar cells, more representative of the small intestine (Pinto *et al.*, 1983). These cells exhibit characteristics typical of those found in absorptive small intestinal epithelial cells *in vivo* (Ranaldi *et al.*, 1992; and see figure 2.1, chapter 2). These features include brush border with well-developed microvilli, tight apical junctions, a polarised distribution of membrane components such as enzymes, receptors, ion channels, and lipid molecules (Grasset *et al.*, 1984), and vitamin uptake (Vincent *et al.*, 1985). In addition, several active transport systems that are located in small intestinal cells, such as those for sugars, amino acids, dipeptides, bile acids, and cobalamine intrinsic factor, are also expressed in Caco-2 cells (Wilson *et al.*, 1990; Hu and Borchardt, 1990; Dantzig and Bergin, 1990; and Lahey *et al.*, 1991). Also, the permeability of a substance across Caco-2 monolayers has been shown to give a fair assessment of its *in vivo* absorption regardless of the mechanism of transport (Yee *et al.*, 1997). The presence of tight junctions in

Caco-2 cells can be demonstrated by an inability of permeability markers to pass across the cell monolayers (Wilson *et al.*, 1990).

5.4 The T84 Cell Line

The human colonic epithelial cell line T84, originates from a lung metastasis of a well-differentiated adenocarcinoma (Murakami and Masui, 1980). This cell line grows on permeable supports, soon again becoming well differentiated when reaching confluence (Dharmsathaphorn *et al.*, 1984; and Madara *et al.*, 1987a). The resultant monolayers have a columnar appearance, morphologically resembling foetal colon crypt epithelial cells, with distinct apical and basolateral surfaces. Though these cells do not form well-developed brush borders, they do form tight junctions between cell. The latter has been determined by electrical resistance measurements and the presence of a prominent actin-rich perijunctional microfilament ring, similar to that seen in native intestinal epithelia, which controls tight junction structure (Madara *et al.*, 1988).

In addition, there is evidence to suggest that T84 cells behave like intestinal tissue *in situ* with respect to inflammation. This is because in the lumen and mucosa of patients with inflammatory bowel disease, there are elevated prostaglandin levels (Rampton and Sladen, 1984). Likewise, in tissue and cultured cell preparations, numerous inflammatory mediators have been shown to stimulate the synthesis and/or release of prostaglandins (Lawson and Powell, 1987; Hong and Levine, 1988; and Bern *et al.*, 1989). Pretreatment of T84 cells with indomethacin (1 μM) inhibits such prostaglandin release (Berschneider and Powell, 1992).

5.5 Measurement of Transepithelial Resistance (TER)

When cells are grown on a permeable support they form a monolayer. Measurement of electrical resistance across the cellular monolayer is a relatively sensitive measure of its integrity and permeability (von Bonsdorff *et al.*, 1985). The TER is inversely proportional to paracellular permeability, reflecting resistance predominantly across tight junctions and not the cell membrane (Madara, 1983) since firstly, in leaky epithelia, such as in the intestine, up to 85% of the electrical current flows between the cells via the paracellular route (Frizzell and Schultz, 1972), and secondly, paracellular resistance increases logarithmically with increasing number of junctional strands. Relatively minor alterations in junctional permeability produces substantial changes in the TER, attesting to the high sensitivity of this method of permeability measurement (Madara, 1983). Treatments which disrupt the tight junctions, such as removing Ca^{2+} from the medium (Collares-Buzato *et al.*, 1994) or inhibiting actin filament formation with cytochalasins (Madara *et al.*, 1983), cause the monolayers to lose their electrical resistance properties. Furthermore, changes in the TER will reflect structural as well as functional changes of the tight junctions, whereas electron microscopic evaluation can only detect structural changes (Milton and Knutson, 1990).

TER is generally expressed in $\Omega.cm^2$, which is the resistance measured in Ω multiplied by the area of the filter in cm^2. For Caco-2 monolayers, the TER is usually between 150 and 400 $\Omega.cm^2$ (Grasset, 1984; Hidalgo *et al.*, 1989; and Wilson *et al.*, 1990). This is similar to that of colonic cells (whole rat colon ~ $300\Omega.cm^2$) yet higher than that of small intestinal cells (whole rat small intestine ~ $60\Omega.cm^2$) (Frizzell and Schultz, 1972), indicating the formation of

tighter epithelia. Monolayers of T84 cells typically have an even higher TER of ~1,500 $\Omega.cm^2$ (Madara and Dharmsathaphorn, 1985).

The TER can be measured by several means. The most established technique employs the use of the *Ussing chamber* (Ussing and Zehran, 1951). Cells grown on permeable filters are mounted in between two compartments of the U-shaped chamber, bathed in the appropriate buffer, and resistance measured across the cell monolayer.

Another technique to determine the TER involves using the *Millicell*. This involves passing a potential difference across the cell monolayer using specially designed electrodes, and measuring the resistance.

5.6 Materials and Methods

5.6.1 Materials

The cell lines Caco-2 and T84 were obtained from European Collection of Animal Cell Cultures (ECACC), Salisbury, England.

Gibco BRL, Paisley, Scotland, supplied modified eagles medium (MEM), Dulbecco's modified eagles medium (DMEM), non-essential amino acids (NEAA), l-glutamine, heat-inactivated foetal bovine serum (FBS), trypsin-EDTA (0.25%-1mM), Ca^{2+}/Mg^{2+} -free phosphate-buffered saline (PBS) and trypan blue 0.4% (w/v). ^{51}Chromium-labelled ethylenediaminetetraacetic acid (^{51}CrEDTA) was obtained from Amersham Life Science Ltd, Buckinghamshire, England. The sterile plastic instruments were purchased from Corning Costar, Buckinghamshire, England.

5.6.2 Methods

Maintaining Sterile Conditions

Crucial to all cell culture experiments is the avoidance of microbial infection. This is because the cells, being *in vitro*, have no resistance against such invasion and easily become contaminated, resulting in the loss of function. Therefore most of the plastics used were sterile and disposable and almost everything (including gloves) was sprayed with 70% ethanol before coming into contact with the cells. The use of a class 3 laminar flow cabinet (Holten LaminAir) also helped prevent contamination

Passaging Cells

Passaging was performed in the flow cabinet. When not being used the flask of cells was kept in an aerated (95% O_2 and 5% CO_2) incubator (Heraeus) at 37°C, with the lid of the flask slightly loosened to enable the cells to receive sufficient oxygen and carbon dioxide for growth.

The cells were grown in 10ml prewarmed (37°C) medium. For the Caco-2 cells the culture medium comprised of MEM containing 1% NEAA, 1% glutamine and 20% FBS, and for the T84 cells the medium was DMEM: nutrient mix F12 (1:1) with 1% glutamine and 10% FBS. In both cases, the culture medium was a red-coloured solution since it contained the indicator phenolphthalein red. This medium turns purple when alkaline, such as when the cells have exhausted the nutrients from the medium, and orange when acidic, for example during bacterial contamination.

The cells were examined each day under a microscope to check for confluency and any signs of microbial contamination. Every alternate day, the medium

was poured off and replaced with fresh medium, known as "refeeding". This ensured the cells always had adequate nutrients for growth.

When the cells were confluent they were "passaged" either for experimental use or for propagation. This involved pouring off the culture medium and washing the cells gently with prewarmed PBS three times. 1ml prewarmed trypsin-EDTA was then added. Both Caco-2 and T84 cells attach very strongly to the flask wall so both a protease (trypsin) and a chelating agent (EDTA) are required to detach the cells from the flask wall. The lytic action of trypsin is prevented by the serum in the medium, so it is important for the cells to be washed well for trypsin to work. The flask was reincubated for approximately 2-3 minutes for Caco-2 and 10-15 minutes for T84 cells, to enable the trypsin to work. (T84 cells require relatively vigorous trypsinisation because these cells attach extremely firmly to the culture flask and the cells tolerate it well (Dharmsathaphorn and Madara, 1990)). The side of the flask was then gently tapped to increase detachment.

9ml of culture medium was added to counteract the action of trypsin. By gently pipetting up and down several times, a homogenous suspension of cells was obtained. 1ml of this suspension was transferred to a new flask containing 9ml fresh medium, and this new flask then returned to the incubator until the cells were confluent again. Both Caco-2 and T84 cells take approximately 5 days to reach confluency.

Counting the Cells

When culturing a new cell line, it is practice to count the cells to estimate how many viable cells are present per cm^2 area of attached surface. In short, this involved mixing the cell suspension with equal volumes of trypan blue, and loading a haemocytometer (Improved Nebauer) with 20µl of this

mixture. Viable cells can exclude trypan blue but those which are dead cannot and so take up a distinctive blue coloration (figure 5.2).

Measurement of TER Using the Ussing Chamber

The cell suspension, obtained from passaging, was concentrated down to 2 x 10^6 cells/ml by resuspending in the appropriate volume of medium. 0.5ml of this cell suspension (i.e. 1 x 10^6 cells) was added to the inner (apical) well of the culture plate and 4ml of medium to the outer (serosal) well. The cells were incubated with the wells being refed every other day.

The 12mm diameter, polycarbonate filter (0.4μm pore size) of the inner well was purpose built so that it could be snapped out and assembled in the Ussing chamber (World Precision Instruments, Inc). This was done so with the bottom of the "snapwell" (serosal side) facing the left hand side of the chamber. Both halves of the chamber were filled with 20ml Ringer's solution comprising 114mM NaCl, 5mM KCl, 1.65mM Na_2HPO_4, 0.3mM NaH_2PO_4, 25mM $NaHCO_3$, 1.25mM $CaCl_2.2H_2O$, 1.1mM $MgSO_4.7H_2O$, 10mM D-glucose, pH 7.4 with 1M NaOH. This was then gently oxygenated and the temperature maintained at 37°C by a temperature jacket (figure 5.3).

Electrodes, embedded in 3% agar and 150mM KCl, were connected to both chambers. Resistance (R) measurements were made by clamping the potential difference to a known voltage (V) and measuring the current (I) necessary to produce this deflection. Resistance could then be calculated using Ohm's law (V=IR). Similarly, it was also possible to clamp the current at required values and measure the voltage. The voltage was initially measured at 100μA for approximately 30 minutes to get a stable baseline. The voltmeter allowed for compensation of fluid resistance. That is, by subtracting the resistance measured to pass fluid through the chamber from the value required to pass

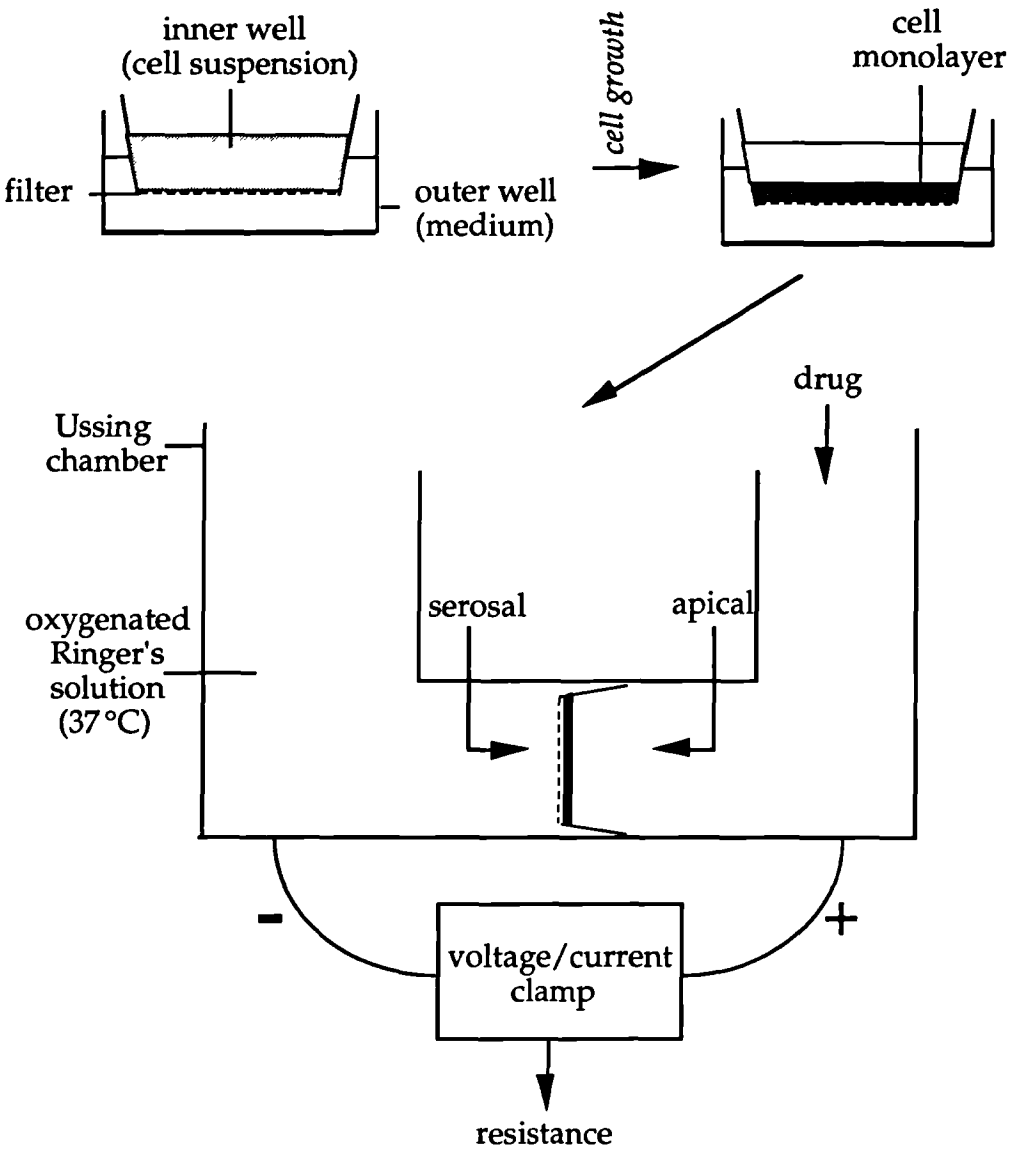


Figure 5.3 Ussing chamber set up

$1x10^6$ cells were plated out onto specialised snapwells. Following cell growth, the snapwells were snapped out of the plate and placed at the bottom of the U-shaped Ussing chamber and a voltage passed across it. Drugs were added to the right hand bath (the apical side of the monolayer), and their effect on the TER are observed.

fluid through when the filter was present, an accurate resistance measurement across the filter is achieved.

Measurement of TER Using the Millicell Electrode

Using 12mm diameter polycarbonate transwells (0.4μm pore size), 0.75ml cell suspension was added to the inner well and 1.5ml medium to the outer well. The cells were incubated for 48 hours in order to attach to the filter.

Before taking a reading, the Millicell-ERS electrode (Millipore (U.K.) Ltd) was immersed in 70% ethanol for 15 minutes, to rid it of any contaminants, air-dried, then dipped in serum-free medium to equilibriate for a further 15 minutes. The two-probed electrode was positioned over the cells such that the longer probe was immersed in the outer well and the shorter probe in the inner well, making sure there was no physical contact with the filter to avoid cell or filter damage. By passing a current between the electrodes, i.e. across the monolayer, the potential difference was automatically measured and the resistance across the cell monolayer calculated, giving a reading in kilo-ohms (kΩ) on the meter (figure 5.4). After obtaining a reading, the cells were refed with fresh medium.

This process of measuring TER then refeeding was repeated every alternate day until a maximum resistance was measured. Preliminary experiments revealed that for Caco-2 cells the average maximum TER was ~800Ω.cm^2 and was achieved after approximately 17 days, and for T84 cells it was ~1200Ω.cm^2 after approximately 7 days. It is worth noting that the time it took for the cells to give maximum TER in the transwell plate did not correlate with the time it took for them to reach confluency in the culture flasks. Thus, the progressive rise in epithelial resistance most likely reflects the continued maturation and formation of tight junctions rather than progressive cell confluence (Ma *et al.*,

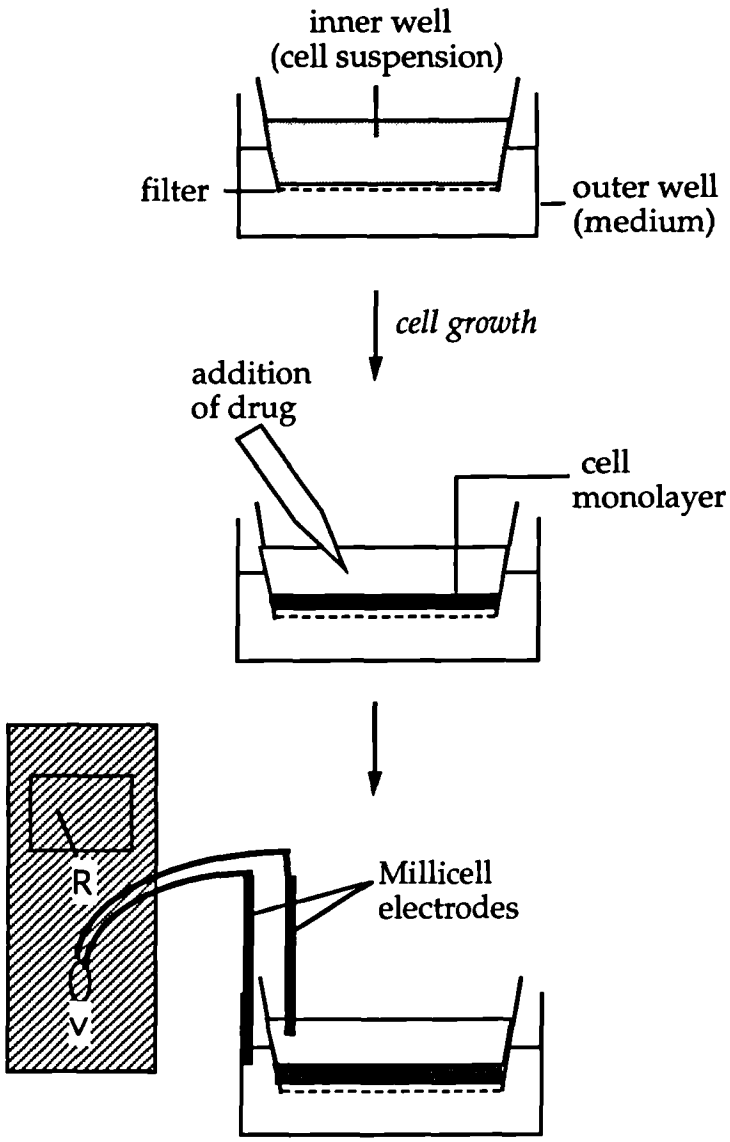


Figure 5.4 Millicell set-up

1.4×10^6 cells were seeded onto transwells and grown for 2 days. The Millicell electrodes were then appropriately positioned in the wells and a potential difference passed across the electrodes, giving a resistance reading in kilo-ohms of the cell monolayer. The monolayers were then replenished with fresh medium and the process of measuring their resistance and refeeding was repeated every other day.

1992).

Therefore, subsequent experiments looking at NSAID effect on the TER were performed on T84 cells 7 days after seeding onto transwells, since its greater TER meant that drug effect would be easier to detect than in Caco-2 cells. If the time-interval between each reading was greater than 5 minutes, the plate was returned to the incubator to ensure that the temperature of the medium did not drop below 37°C.

5.7 Aims of the Studies

Ussing Chamber Experiments

Experiment 1 - Resistance over varying currents

Ideally, the resistance across the monolayer should remain constant with varying current. This was tested by clamping the current at set values and calculating the resistance (in ohms). This was carried out in Caco-2 cells 2, 3, 6 and 7 days after seeding onto the wells. The effect of indomethacin on the TER of 2, 3 and 7 day old cells was then studied by adding 0.125mM indomethacin to the apical side of the cell monolayer (RHS chamber). In the 6 day old Caco-2 cells, the effects of 0.015 and 0.045mM dinitrophenol were observed.

Experiment 2 - Resistance over time

A decrease of TER would indicate a loosening of the tight junctions. The TER was measured over time to assess firstly how long the monolayers retained their junctional integrity for a certain length of time, and secondly how long it took for indomethacin to have an effect on the TER. This was carried out by clamping the current at 100μA and measuring the change in resistance over

time in 4, 5, 13, 14 and 17 day old Caco-2 cells, and also in 8, 9 and 10 day old T84 cells. In the 4 day old Caco-2 cells the effect of 0.125 and 0.375mM indomethacin was tested and found to have very little effect. Therefore, in the remaining groups of cell monolayers a higher concentration of indomethacin (2.5mM) was used.

Millicell Electrode Experiments

Study 1 - Effect of indomethacin on TER of T84 cells

The effect of 0.5 to 2mM indomethacin was tested on the cells at regular intervals over 6 hours then again 24 hours later. This was done by adding 7.5, 15, 22.5 and 30µl of 50mM indomethacin (made up in 10% DMSO) to the inner well, then measuring the TER at the set time time-points.

Study 2 - Effect of 10% DMSO on TER of T84 cells

In order to rule out a dilution effect caused by adding different volumes of indomethacin or DMSO, experiment 1 was repeated but with (a) 10% DMSO added to the wells at the same volumes as indomethacin and also with (b) 7.5µl of different strengths of DMSO.

Study 3 - Effect of NSAIDs (same volume, different stocks)

It was then decided to keep the volume of drug added constant throughout to rule out dilution of the medium having an effect on the TER. Therefore, indomethacin and aspirin were now prepared in different strengths of DMSO and 7.5µl of the different stocks added to give a final concentration range from 0.05 to 2.5mM for indomethacin and 0.001 to 1mM for aspirin. Similarly, different stock solutions of naproxen were made up in water so that addition of 7.5µl gave a concentration range of 0.0001 to 1mM. (7.5µl water was found to have no effect on the TER).

Study 4 - Effect of DNP on TER of T84 cells

Different stock solutions of DNP were made up in 0.5% ethanol. Either 7.5 or 15µl was added to the cells to give a final concentration range of 0.001 to 0.04mM, and the resistance measured over time. Corresponding volumes of only 0.5% ethanol were also tested.

Study 5 - Effect of pH on TER of T84 cells

Maintaining a pH of 7.4 in the bathing medium is important since alterations in pH will alter cell growth and an alkaline pH, particularly, will markedly reduce cell viability (Wilson, 1987). In the previous experiments, although all the original drug solutions were adjusted to pH 7.4 with 1M NaOH, the cell culture medium itself was not buffered. To investigate if the TER changes previously seen with the drugs were as a result of pH changes in the medium following drug addition rather than a drug effect *per se*, the effect of pH on the TER was tested by adding different volumes of 1M HCl or 1M NaOH to the cells to vary the pH between 4 and 10.

Study 6 - Effect of indomethacin in HEPES-buffered medium

Due to their being an effect of pH changes on the TER, HEPES-buffered medium was used for all subsequent experiments. The effect of indomethacin (0.2 to 1mM) on the TER was measured in this new medium at regular intervals between 5 and 240 minutes. It was found that by the first reading at 5 minutes, the TER of the cells was already significantly reduced by indomethacin. Therefore, in order to follow this decrease more closely, the TER was then measured at 5 second intervals until 30 secs, then at 60 secs, then every minute until 5 minutes had elapsed.

Study 7 - Effect of aspirin and naproxen on TER of T84 cells

A range of concentrations of aspirin between 0.4 and 20mM and naproxen between 1 and 10mM were used, and the TER measured over time.

Study 8 - Effect of misoprostol on TER of T84 cells

A range of concentrations of misoprostol from 0.4 to 4mM were used, to see the effect of this prostaglandin derivative on the TER.

Study 9 - Effect of naproxen on ^{51}CrEDTA flux

As well as measuring the TER, addition of hydrophilic permeability markers such as ^{51}chromium-labelled ethylenediaminetetraacetic acid (^{51}CrEDTA) to cellular monolayers will enable paracellular permeability to be measured. Therefore, to the inner well was added either (a) 10mM naproxen with 30µl ^{51}CrEDTA (3µCi), (b) naproxen alone or (c) ^{51}CrEDTA alone. Then at regular intervals for 4 hours, the resistance was measured. Then the contents of the inner and outer wells transferred to scintillation vials in order to measure their respective counts per minute (cpm) using a gamma counter (Wallac, LKB 1262). An increase in the cpm of the outer well and a decrease in the inner well, would suggest ^{51}CrEDTA flux through the monolayer by a loosening of intercellular tight junctions.

Study 10 - Effect of NSAIDs pre-dissolved in the medium

Up till now, all the drugs tested had been added directly to the cells. Often duplicate experiments gave different results. These discrepancies may have arisen from the 'location' the drug was pipetted with respect to the cells, as many of the drug solutions were fairly viscous and tended not to disperse readily in the medium. (It was not possible to use a plate shaker to disperse the drug solution due to the fragility of the cells). Thus, if the drug solution was pipetted very near to the monolayer, the cells would be exposed to a

higher (more toxic) concentration sooner than if the drug was pipetted near the top of the well (see figure 5.4). For this reason it was decided to prepare the drugs indomethacin and naproxen as before, then to mix the required volume to 750µl prewarmed medium in a sterile vial rather than add to the well directly. Then, in order to see the effect of the drug on the TER, the pre-existing medium from the inner well was removed and replenished with the freshly-prepared drug-treated medium.

5.8 Results

5.8.1 Ussing Chamber Results

Experiment 1 - Resistance over varying currents

The Caco-2 cells gave a TER between 10 and 100 $\Omega.cm^2$. Such readings were much lower than expected and did not correlate with the age of the cells. However, the TER remained fairly constant with increasing current.

Addition of 0.125mM indomethacin did not bring about any significant change in the TER. In the 3 day old Caco-2 cells (figure 5.5a), there appeared to be a 30% drop in TER after a 30 minute incubation with indomethacin. However, results with the 7 day old cells (figure 5.5b), revealed that this decrease occurred in untreated cells as well and, therefore, probably represented loss of cellular integrity after 30 minutes rather than a drug effect. This was substantiated in the 2 day old cells (figure 5.5c), when after indomethacin had been washed out and the cells left for one hour, a decrease in the TER was again observed. The suggestion is that the cells do not remain viable in the chamber after 30 minutes.

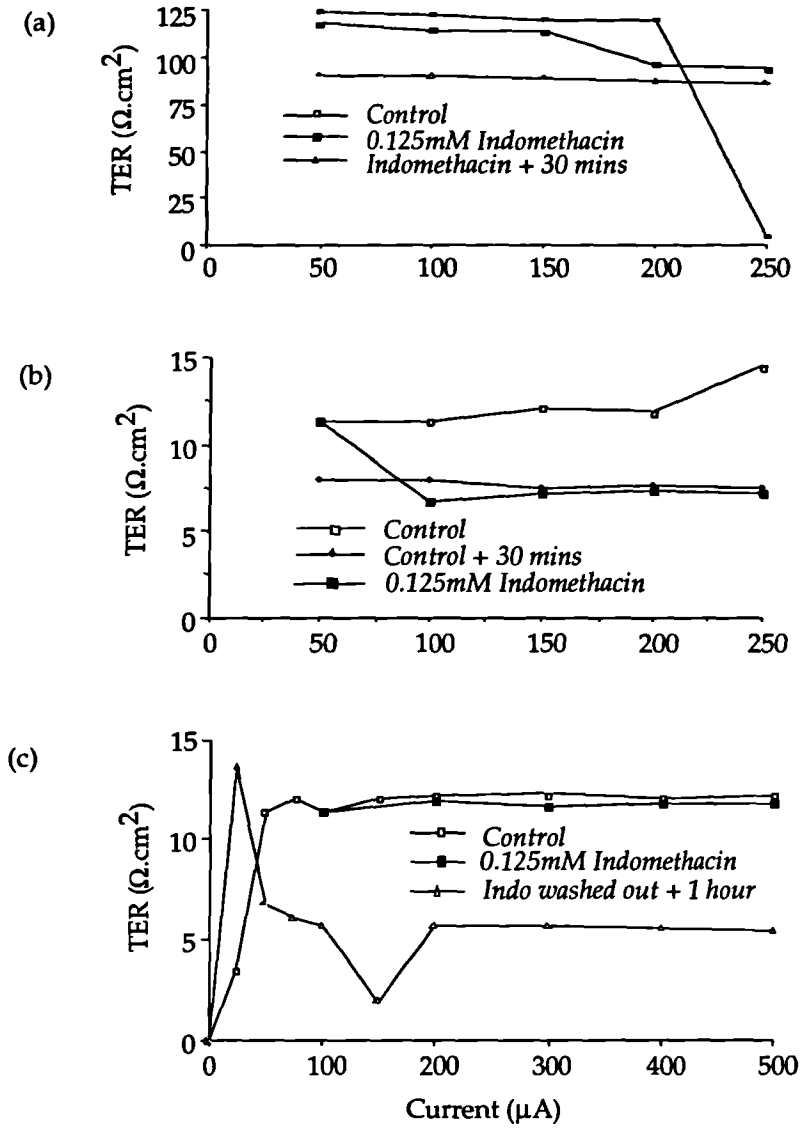


Figure 5.5 Effect of indomethacin on TER of Caco-2 cells over increasing current using the Ussing chamber

$1x10^6$ Caco-2 cells were grown in snapwells for a period of (a) 3, (b) 7 and (c) 2 days before being assembled in the Ussing chamber. The TER (expressed as $\Omega.cm^2$ = resistance measured x area of the filter) of untreated (control) or treated (0.12mM indomethacin) cells was measured over increasing current. Each data point represents one experiment.

0.015 or 0.045mM DNP did not appear to affect the TER of 6 day old Caco-2 cells (figure 5.6). As with indomethacin, when the cells had been washed free of drug, there was a marked decrease in the TER. It was found that control resistance measurements were not consistent between preparations.

Experiment 2 - Resistance over time

In Caco-2 (figure 5.7) and T84 (figure 5.8) cells, there was a consistent decline in the TER over time, irrespective of the age of the cells. Addition of indomethacin did not further reduce the TER but showed the same pattern of decrease over time, indicating that this was not a drug effect but due to loss of cellular viability.

5.8.2 Millicell Results

Study 1 - Effect of indomethacin

Figure 5.9 shows that concentrations of indomethacin between 0.5 and 2mM decreased the TER of T84 cells. The highest concentration of indomethacin used, 2mM, caused the maximum decrease to approximately 35% of control.

With all the concentrations used, the initial sudden drop in the TER was followed by a gradual rise over 30 minutes and then a decrease again. As TER measurements were done every 5 minutes for the first half an hour, it was unfeasible to return the plate to the incubator in between readings. Consequently, the increase in TER seen over this time period may actually correspond to the temperature of the cells falling below room temperature and not a drug effect *per se*, since a similar rise in the TER was seen in untreated cells (see discussion).

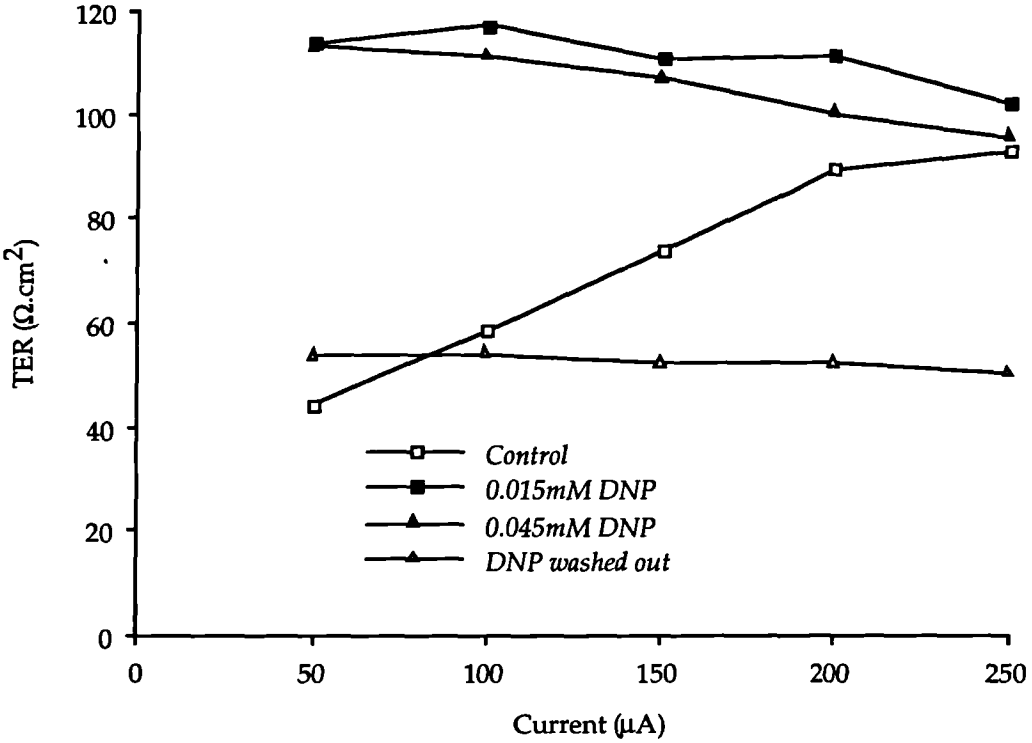


Figure 5.6 Effect of DNP on TER of Caco-2 cells over increasing current using the Ussing chamber

$1x10^6$ Caco-2 cells were grown in snapwells for a period of 6 days then placed in the Ussing chamber. The TER of cells treated with DNP (0.015mM or 0.045mM) or untreated cells (control) was monitored over increasing current. Each data point represents one experiment. TER was expressed as $\Omega.cm^2$ (resistance measured multiplied by the area of the filter).

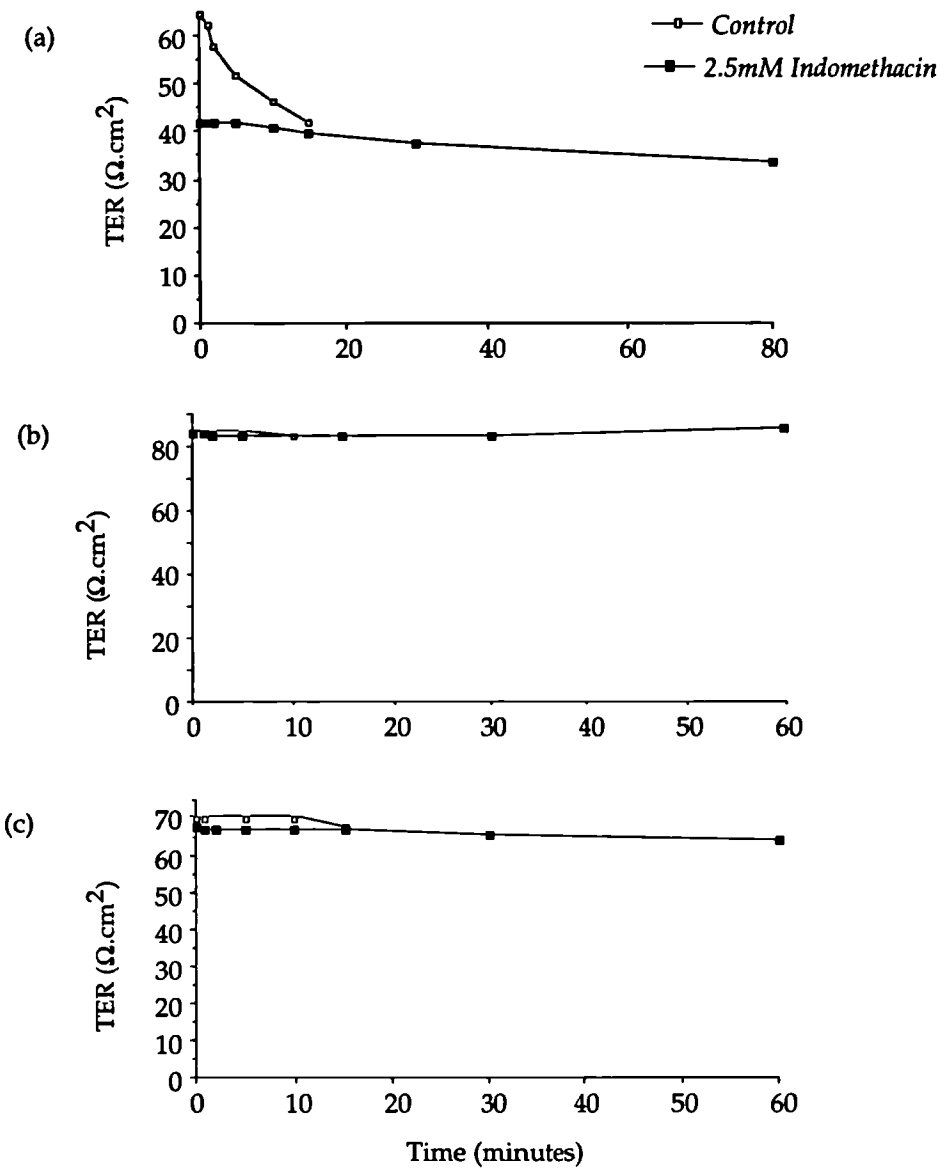


Figure 5.7 Effect of indomethacin on TER of Caco-2 cells over time using the Ussing chamber

1 x 10^6 Caco-2 cells were seeded onto snapwells and grown for (a) 13, (b) 14; or (c) 17 days. The filters were then assembled into the Ussing chamber and 2.5mM indomethacin added to right-hand bath feeding the apical side of the monolayer. Control monolayers were untreated. The TER was then measured over time. TER is expressed in $\Omega.cm^2$ (resistance x area of filter). Each point represents 1 experiment.

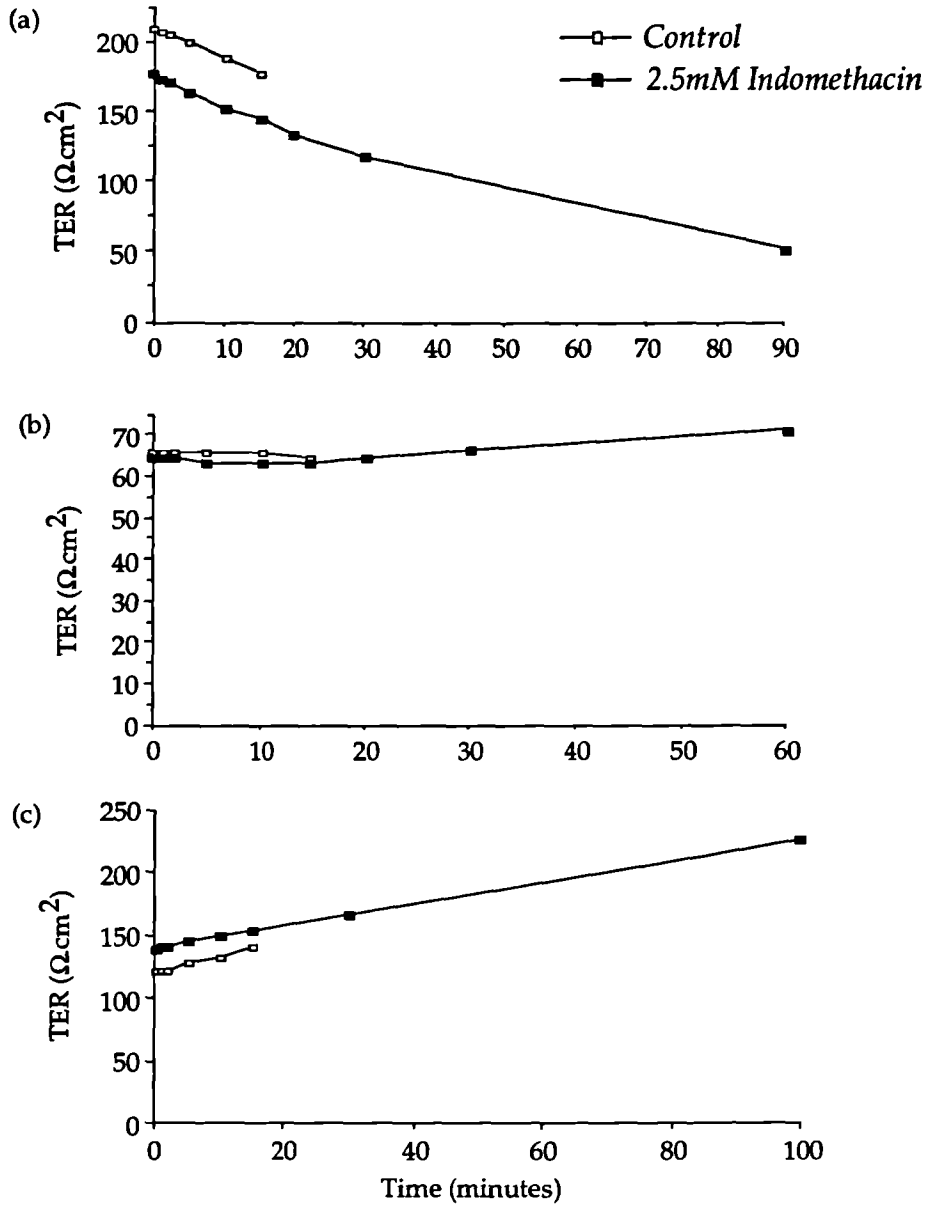


Figure 5.8 Effect of indomethacin on TER of T84 cells over time

1.4 x 10^6 T84 cells were plated out in snapwells for a period of (a) 8; (b) 9; and (c) 10 days then positioned in the Ussing chamber. The cells were either treated with 0.25mM DNP by adding to the right hand half of the chamber or untreated (control). Their TER was then observed over time. TER was expressed as $\Omega.cm^2$ (resistance measured multiplied by the area of the filter). Each data point represents one experiment.

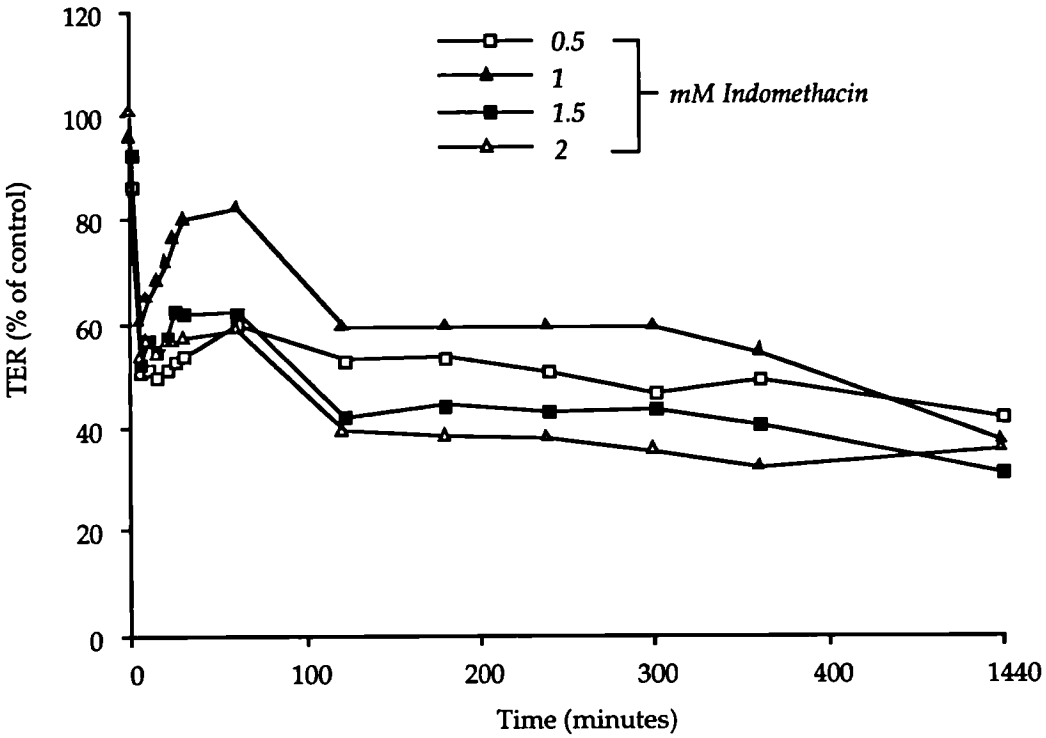


Figure 5.9 Effect of indomethacin on TER of T84 using the Millicell

1.4 x 10^6 T84 cells were grown in transwells for 1 week. Using the Millicell electrode the TER of the monolayers were monitored every alternate day before being refed, until their TER had reached ~1000Ω.cm^2. The monolayers were then treated with 0.5 to 2mM indomethacin and the TER measured at set time-points and compared to control. The TER was expressed as percent of (resistance in presence of drug / resistance in absence of drug). Each point represents the mean of 2 experiments.

Study 2 - Effect of 10% DMSO

Figure 5.10a shows that different volumes of 10% DMSO between 7.5 and 30µl, all appeared to give a TER greater than 100%. Figure 5.10b shows that when 7.5µl DMSO of different strengths was used, the TER remained at approximately 100%, indicating this volume of the solvent (irrespective of final concentration) had no effect on the TER and so would be favourable to use for experiments.

Study 3 - Effect of NSAIDs (same volume, different stocks)

Figure 5.11a shows how over approximately 24 hours, 0.005 and 0.05mM indomethacin had a negligible effect on the TER. 0.5mM reduced TER by a maximum of 50%, whereas 5mM indomethacin decreased the TER by 95% within 5 minutes. Therefore, the effect of indomethacin on the TER appears to be concentration-related.

From figure 5.11b it can be seen that 0.0001 to 1mM aspirin had very little effect on the TER. Over 6 hours, the TER remained at approximately 100% of control.

Figure 5.11c shows that over a concentration range of 0.0001 to 1mM, naproxen did not reduce the TER by more than 30%. The decrease was not related to the concentration of the drug since maximum decrease was obtained by 0.01mM naproxen.

Study 4 - Effect of DNP

There was very little effect of 0.002 to 0.04mM DNP, or of corresponding volumes of its solvent 0.5% ethanol, on the TER. This can be seen in figure 5.12 where the TER remains around 100%.

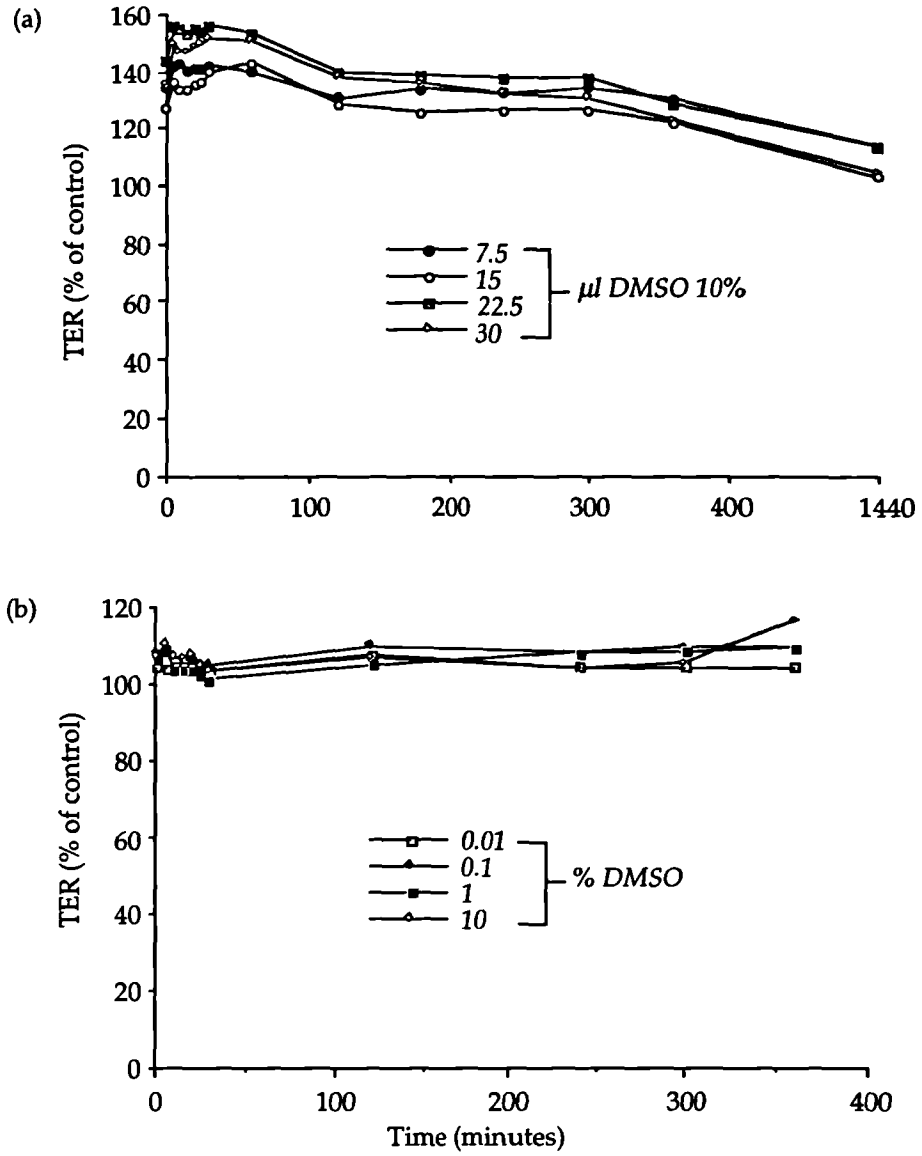


Figure 5.10 Effect of DMSO on TER of T84 cells using the Millicell

1.4 x 10^6 T84 cells were seeded onto transwells. After growing for approximately 1 week, the TER had reached ~1000Ω.cm^2 as determined by the Millicell electrode. The cells were then treated with (a) different volumes of 10% (v/v) DMSO or (b) 7.5µl of different strengths of DMSO. The TER of the monolayers was monitored over time and compared to untreated cells (control). TER is expressed as percent of (resistance in presence of DMSO / resistance in absence of DMSO). Each point represents mean of 2 experiments.

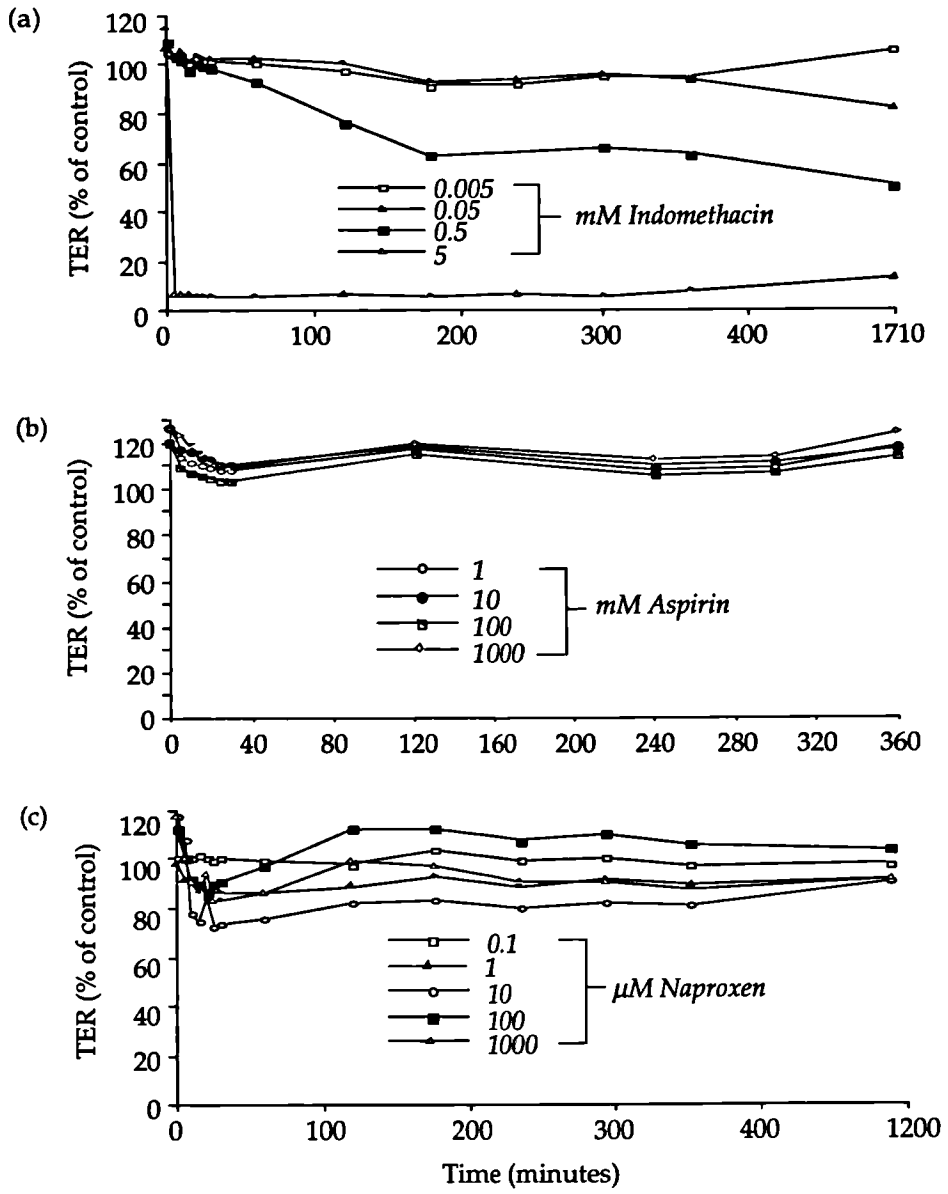


Figure 5.11 Effect of NSAIDs on TER of T84 cells using the Millicell

When 1.4 x 10^6 T84 cells were grown in transwells for 1 week they gave a TER of ~1000Ω.cm^2 when measured by the Millicell electrode. The monolayers were then incubated with 7.5µl of different stocks of (a) indomethacin, (b) aspirin or (c) naproxen. The TER of the cells were observed at various times after treatment and compared to control (untreated cells). TER expressed as percent of (resistance in presence of drug / resistance in absence of drug). Each point represents mean of 2 experiments.

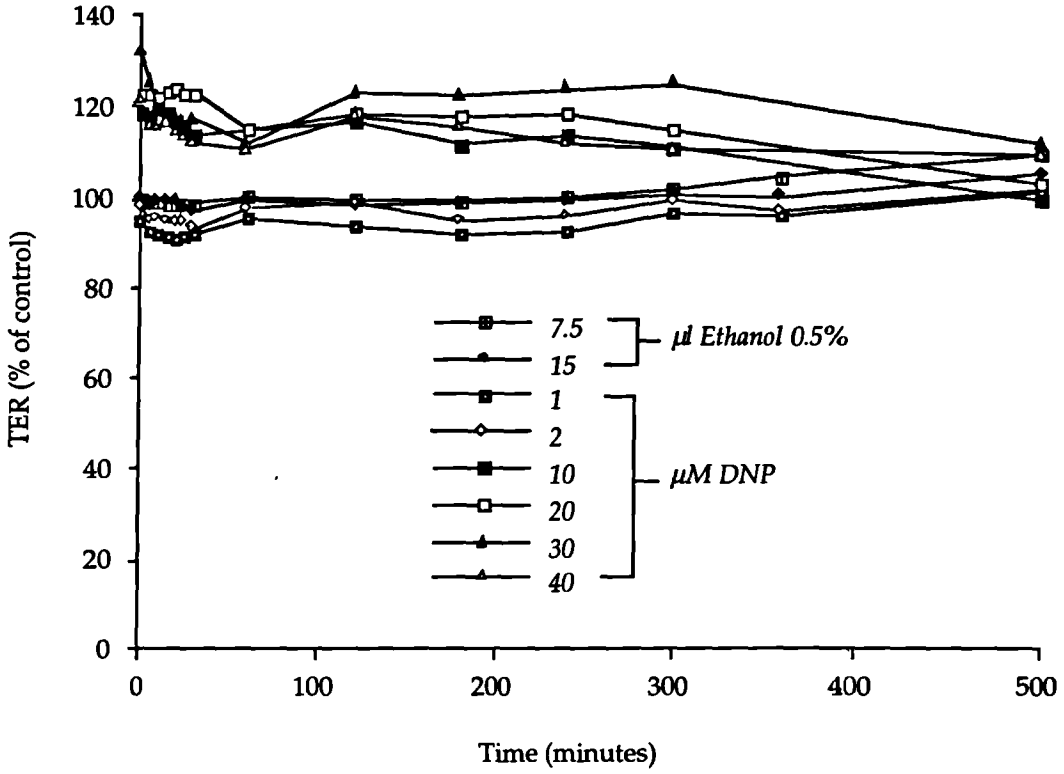


Figure 5.12 Effect of DNP on TER of T84 cells using the Millicell

1.4 x 10^6 T84 cells were grown in transwells. Using the Millicell electrode, the TER of the monolayers was monitored every alternate day before being refed, until their TER had reached ~1000Ω.cm^2. The cells were then treated with 7.5µl of different stocks of DNP and their TER measured at various time points and compared to untreated cells. TER expressed as percent of (resistance in presence of drug / resistance in absence of drug). Each point represents mean of 2 experiments.

Study 5 - Effect of pH

The effect of different pH's on the TER can be observed in figure 5.13. In the currently used unbuffered medium, a pH of 4 to 7 and pH 10, gave TER values below that of control, with pH 10 reducing the TER the most. A pH of 8 gave a TER similar to the control value, indicating at this pH the cell viability would not be compromised.

However, even with the drugs being prepared at pH 7.4, their addition to the medium may still change the pH slightly. Therefore, a culture medium buffered with HEPES was subsequently used. (The existence of such a medium prior to starting the experiments was unknown and previously published reports have not mentioned pH changes as a problem).

Study 6 - Effect of indomethacin in HEPES-buffered medium

Figure 5.14a show that 0.2 to 1mM indomethacin significantly decreased the TER within 5 minutes of addition. 10% DMSO had no effect.

Following the decrease in detail over 5 minutes, it can be seen from figure 5.14b that the decrease is time-dependent. The reduction is also related to the concentration of indomethacin since at 5 minutes the TER (% of control) by 0.2mM indomethacin is 60%, 0.5mM is 30%, and 1mM is 18%.

Study 7 - Effect of aspirin and naproxen in HEPES-buffered medium

Figure 5.15a shows the effect of aspirin on the TER (in HEPES-buffered medium). Only the highest two concentrations 10 and 20mM significantly reduced the TER to approximately 60% of control. The lower concentrations had little or no effect on the TER, since the TER was around 100%.

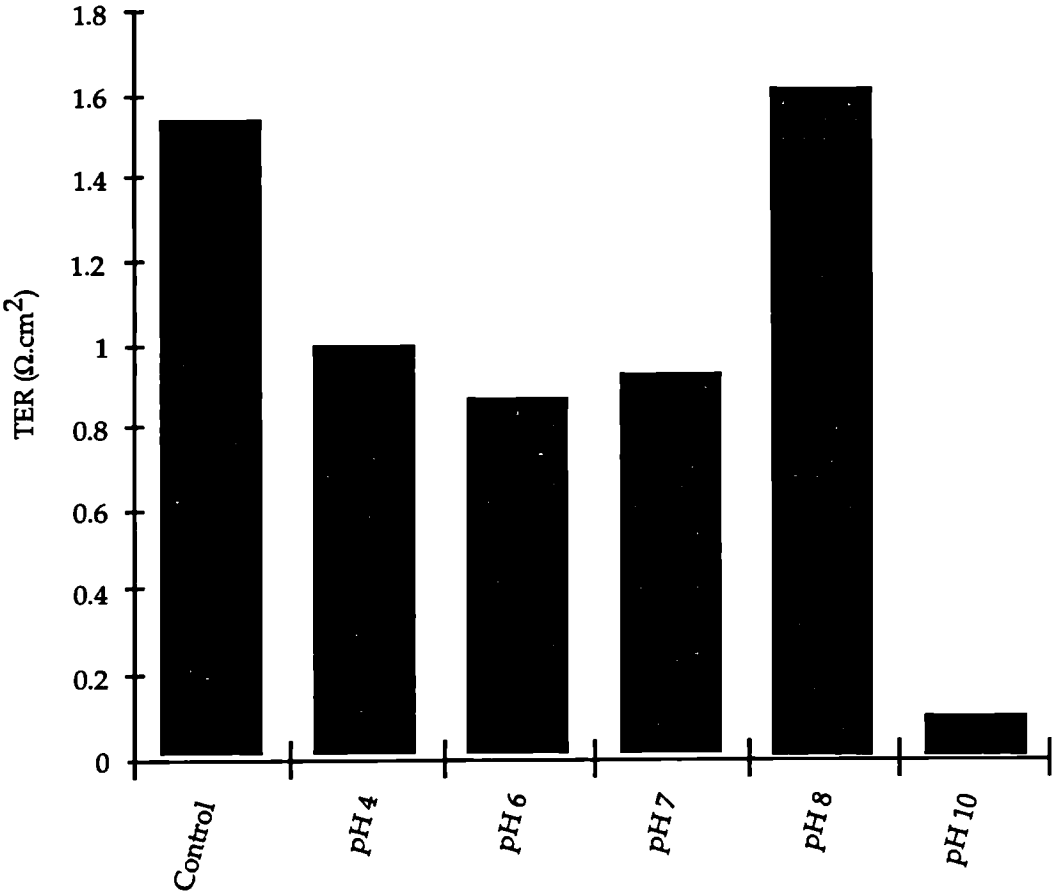


Figure 5.13 Effect of pH on TER of T84 cells

1.4×10^6 T84 cells were plated out in transwells and grown until the TER of the monolayers had reached ~1000Ω.cm^2 as asceratined by the Millicell electrode. Using 1M HCl or 1M NaOH, the pH of the inner well was varied as shown, and the TER was measured after a 30 minutes incubation period. The TER was expressed as Ω.cm^2 (resistance measured multiplied by the area of the filter). Each result represents 1 experiment.

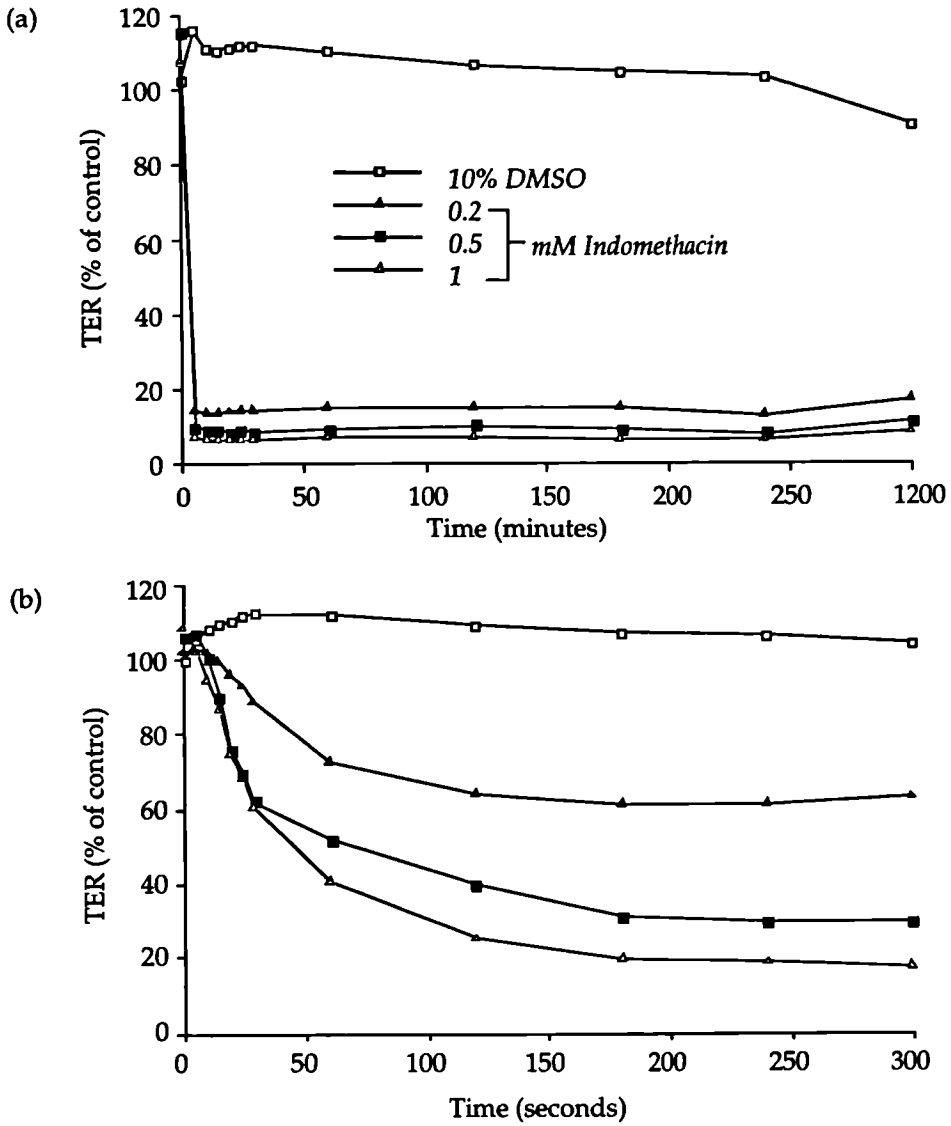


Figure 5.14 Effect of indomethacin on TER of T84 cells using the Millicell
1.4×10^6 T84 cells were plated out in transwells using HEPES-buffered culture medium. After 1 week, the TER of the monolayers was ~1000Ω.cm^2 when measured by the Millicell electrode. 0.2 to 1mM indomethacin was then added to the monolayers and their TER monitored over either (a) 1200 minutes or (b) 300 seconds and compared to untreated cells. TER expressed as percent of (resistance in presence of drug / resistance in absence of drug). Each point represents mean of 2 experiments.

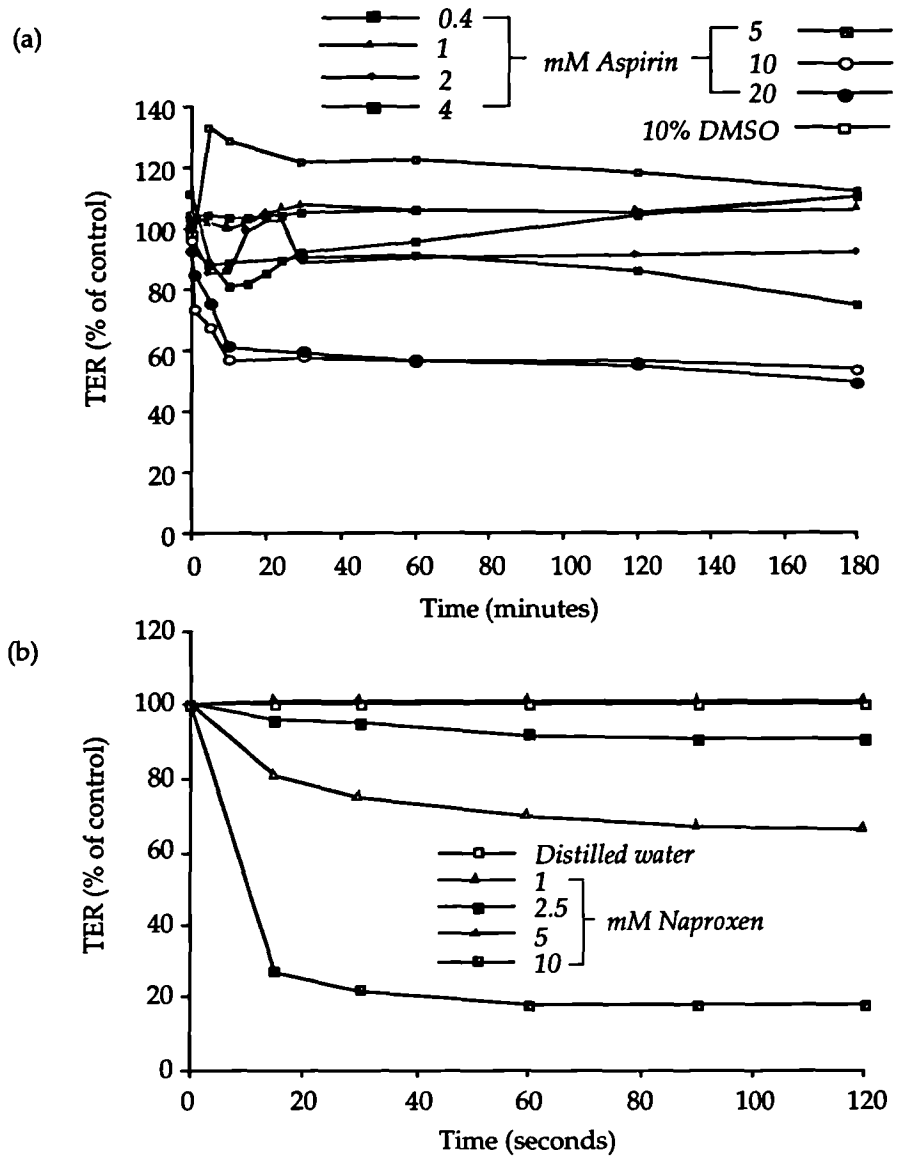


Figure 5.15 Effect of aspirin and naproxen on TER of T84 cells using the Millicell

In HEPES-buffered culture medium, 1.4 x 10^6 T84 cells were seeded onto transwells. These were grown until their TER had reached ~1000Ω.cm^2 as ascertained by the Millicell. The monolayers were then incubated with (a) aspirin or (b) naproxen at the concentrations indicated. The TER of the cells was measured at various time intervals and compared to untreated cells. TER is expressed as percent of (resistance in presence of drug / resistance in absence of drug). Each point represents the mean of 2 experiments.

In figure 5.15b, it can be seen that both the solvent (water) and the lowest concentration of naproxen used (1mM), had no effect on the TER over 2 minutes. At the higher concentrations, maximum reduction in the TER was achieved more or less within 30 seconds, the greatest decrease being caused by the highest concentration of naproxen (10mM) to 26% of control.

Study 8 - Effect of misoprostol

From figure 5.16 it can be seen that 0.4 to 4mM misoprostol did reduce the TER by a maximum of 35%. However, this decrease was not concentration-related since the maximum reduction was seen with 2mM misoprostol.

There was a rise in the TER above 100% with water (the solvent of misoprostol) in the first 10 seconds of readings. This again may have corresponded to a temperature-effect since the water had not been prewarmed to 37°C, therefore causing the temperature of the cells to drop below 37°C.

Study 9 - Effect of naproxen on $^{51}Cr.EDTA$ flux

The TER of T84 monolayers treated with ^{51}CrEDTA alone, remained around 100% of control over 4 hours (figure 5.17), suggesting this marker did not adversely affect cell function during this time. When the radioactivity of the outer well was compared to total radioactivity as a percentage to measure ^{51}CrEDTA flux, it was seen that virtually no ^{51}CrEDTA passed through the monolayer. On addition of naproxen, the TER was decreased within 30 minutes (as seen previously). There was also a time-dependent increase in ^{51}CrEDTA flux through the monolayer, observed as a gradual rise in the outer well cpm (% of total count), over 4 hours.

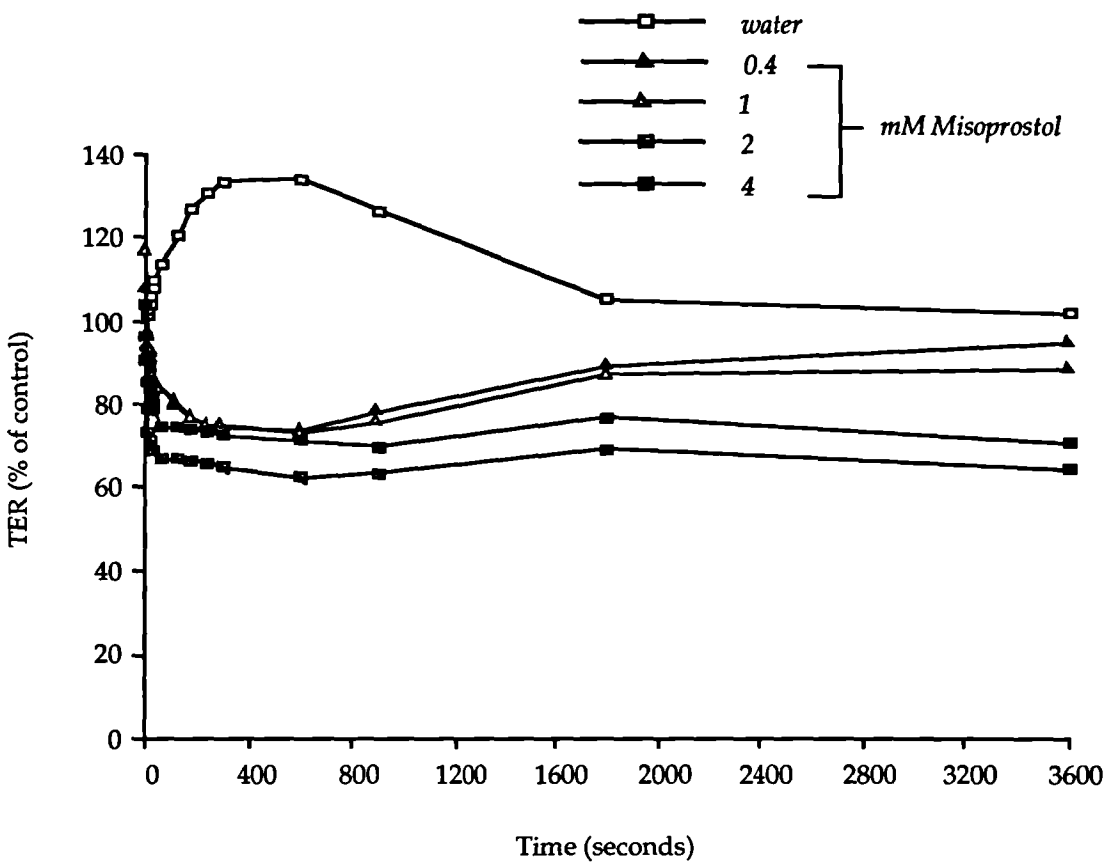


Figure 5.16 Effect of misoprostol on TER of T84 cells using the Millicell
1.4 x 10^6 T84 cells were grown in transwells using HEPES-buffered culture medium. Using the Millicell electrode, the TER of the monolayers was monitored until a value of ~1000Ω.cm^2 had been obtained. These were then incubated with 0.4 to 4mM misoprostol and the TER observed over 3600 seconds and compared to untreated cells. TER expressed as percent of (resistance in presence of drug / resistance in absence of drug). Each point represents mean of 2 experiments.

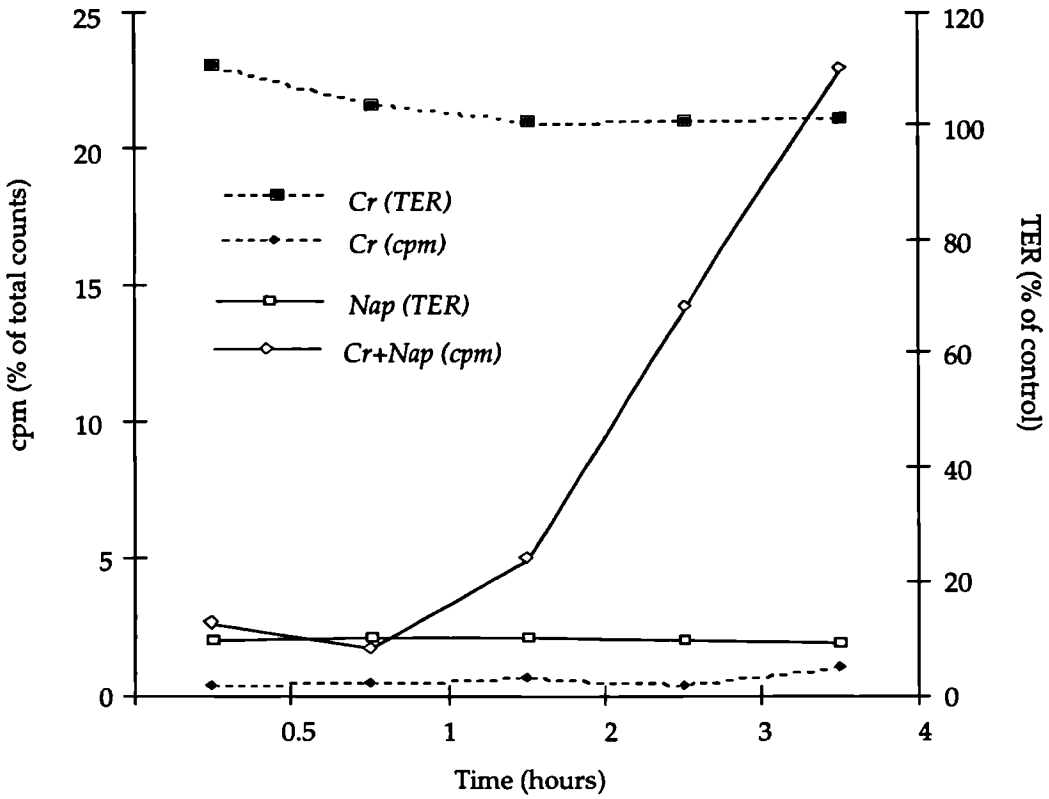


Figure 5.17 Effect of naproxen on the ^{51}CrEDTA flux of T84 cells

In HEPES-buffered culture medium, 1.4 x 10^6 T84 cells were grown in transwells for approximately 1 week so that their Millicell TER reading was ~1000Ω.cm^2. The monolayers were then incubated with 3μCi ^{51}CrEDTA (Cr) in the presence or absence of 10mM naproxen (Nap). The TER of the cells was then observed over 4 hours and compared to untreated cells as a percentage. At the same time-points, the counts per minute (cpm) of the outer and inner wells were also measured using a gamma counter, to assess ^{51}CrEDTA flux. This was expressed as a percentage of the cpm of the outer well over total cpm. Each point represents mean of 2 experiments.

Study 10 - Effect of drugs dissolved in the medium

The effects of replacing the culture medium in the wells with indomethacin- or naproxen-treated medium can be seen in figures 5.18a and b respectively. In both cases, the reduction in the TER by the drugs can be observed gradually over 4 hours, rather than occurring within minutes as happened when the drugs were added directly to the wells (see figures 5.14 and 5.15b).

5.9 Discussion

5.9.1 Optimising Experimental Conditions

The use of intestinal cell lines to monitor the effects of NSAIDs would, in theory, be highly preferable to whole tissue because altered prostaglandin production has a major effect on blood flow and the effect of this is not a problem in cell lines. Therefore, one can observe the effects of NSAIDs on tight junctions in isolation of their effects on blood vessels. However, as the experiments of this chapter demonstrated, getting the right technique and conditions is very important in avoiding artefactual results.

The Ussing chamber was initially introduced to distinguish between active and passive ion movement by using a short-circuit current technique, i.e. voltage-clamping (Ussing and Zehran, 1951). It has since been adapted for the investigation of transport of many markers, being most commonly used in gastrointestinal pharmacology (Grass and Sweetana, 1988). It has the advantage of allowing continuous resistance measurement, so that active transepithelial transport of electrolytes can be monitored. However, when adding small quantities of drug, it is fairly 'hit and miss' as to whether the drug can travel all the way down the reaction chamber to the cell monolayer.

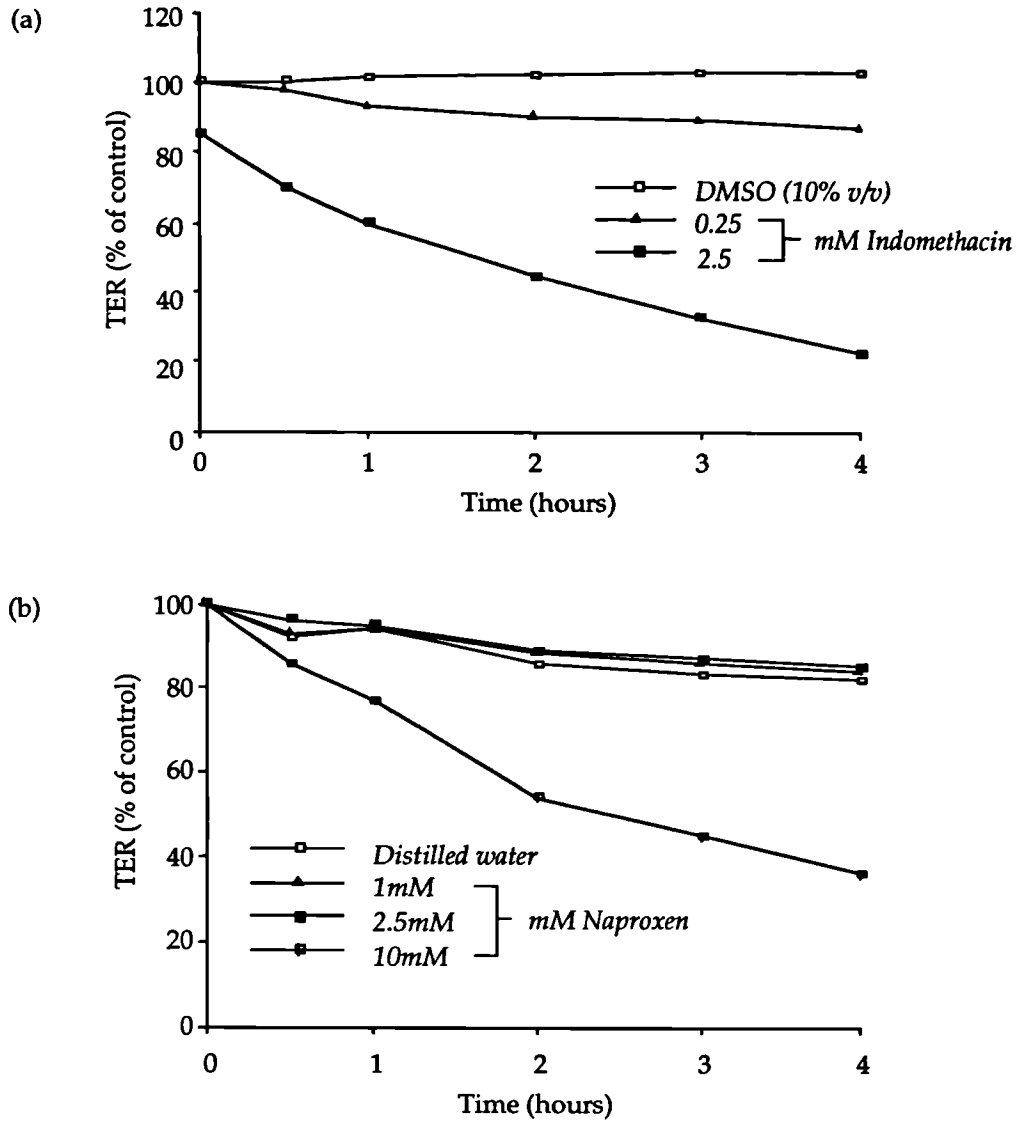


Figure 5.18 Effect on TER of T84 cells when medium replaced with indomethacin- or naproxen-treated medium

Using HEPES-buffered medium, 1.4 x 10^6 T84 cells were grown in transwells until the Millicell TER measurement was ~1000Ω.cm^2. The medium in the wells was then replaced with (a) indomethacin- or (b) naproxen-treated medium at the concentrations indicated. The TER of the cells was observed over 4 hours and compared to untreated cells. TER expressed as percent of (resistance in presence of drug / resistance in absence of drug). Each point represents mean of 2 experiments.

This could have explained the variable results of the present studies. Grass and Sweetana (1988), found that by reducing the volume capacity of the reservoir, quantifying substances with low permeabilities was more accurate.

The present experiments also showed a decrease in the TER over time indicating a loosening of tight junctions. This may have resulted from damage to the cells due to excessive physical manipulation, since the filter had to be removed from its surrounding medium, 'snapped' out of the inner well, then positioned in the chamber. Moreover, the cells were bathed in Ringer's solution and not their original culture medium, perhaps another reason why their viability was challenged.

This lack of success in attaining adequate TER with the Ussing chamber was, however, in sharp contrast to the studies carried out by Madara and colleagues, who consistently reported a TER with T84 cells of ~1,500$\Omega.cm^2$ (Madara and Dharmsathaphorn, 1985; and Dharmsathaphorn and Pandol, 1986). The discrepancy may have arisen from the fact that the latter group worked on cells grown on collagen-coated filters, which increases the ability of T84 cells to attach and confluently spread over the substrate surface immediately after plating, as compared to uncoated filters (Madara *et al.*, 1987b). The advantage of collagen-coated filters was also seen with Caco-2 cells, which reached confluency more rapidly and achieved higher cell densities in, the same length of time, than those on uncoated filters as reported by Grasset *et al.* (1984). This suggests that the collagen matrix may promote cell attachment as well as cell growth (Hidalgo *et al.*, 1989). However, when Hilgers *et al.* (1990) compared Caco-2 cell monolayers grown in the absence to those in the presence of collagen, they concluded that the collagen matrix was unnecessary for the establishment of confluent monolayers. Moreover, the collagen appeared to promote cell migration through the filter

at earlier times in culture, resulting in functional depolarisation of the monolayer. Artursson (1990) even demonstrated a higher TER of Caco-2 cells grown on uncoated filters than that previously reported for collagen-coated filters (Hidalgo *et al.*, 1989). The major discrepancy is that filters of 3μm pore size were used in the studies where collagen was reported to be essential, whereas the pore size was 0.4μm in those studies where collagen was not necessary. Perhaps, therefore, collagen facilitated the formation of confluent monolayers by covering the relatively large pores (Artursson, 1990).

Furthermore, in the studies of Madara's team, the Ussing chamber was modified such that the water turbulence created by oxygenation of the Ringer's solution was minimised, thereby enabling the monolayer to remain intact for more than 2 hours. Pressure to the monolayer was avoided by not clamping the filter into the chamber, rather growing the cells directly on Lexan rings with a collagen-coated Nitex mesh support, which serves as a component of the Ussing chamber itself (Dharmsathaphorn *et al.*, 1984). The Lexan rings, made of rubber, also ensured no damage occurred to the edge of the monolayer. Grasset *et al.* (1984), reduced edge damage by using silicone paste.

Nevertheless, for the purpose of these studies, the Millicell electrode was preferred to the Ussing chamber since the TER could be measured in the original growing environment of the cell monolayers without requiring movement of the cells. Also, with the Millicell, the TER of the cells was higher and it gave reproducible results, indicating the cell monolayer was not greatly perturbed by this means of measurement. However, this technique gave only static measurements, so drug effect could only be monitored at set time points and not continuously.

Another disadvantage was that measurement by the Millicell electrode needed to be performed at room temperature, so there was a risk that the temperature of the culture medium would fall below 37°C. The drop in medium temperature was observed as a steady rise in the TER. A similar finding has been reported previously in Caco-2BBe monolayers (Menconi *et al.*, 1997) and by other investigators (Madara, 1983; and Riehl and Stenson, 1994), where the TER increased at 4°C, possibly as a result of temperature-dependent closing of tight junctions. Likewise in Madin-Darby canine kidney (MDCK) monolayers, a drop in the temperature from 37°C to 3°C resulted in a 306% increase in the TER (Gonzalez-Mariscal *et al.*, 1984). However, the suggestion that the sealing capacity of the tight junctions increased with a fall in temperature, was refuted by the absence of morphological changes. In addition, paracellular transport of fluorescein isothiocyanate (FITC) and mannitol in Caco-2 cell monolayers, showed very little change when the temperature fell below 37°C (Cogburn *et al.*, 1991). Perhaps, therefore, this increase in TER with decrease in temperature is not a tight junctional effect, rather an effect on cell physiology.

The problem then arose of optimising the conditions for measurement of drug effect on the TER by the Millicell. Firstly, as the inner well volume was only 750µl, it was important not to dilute it too much by the addition of drug solutions. Therefore, the drug were prepared in stock solutions of increasing strengths, and 7.5µl added to each well. Any change in TER was, therefore, due to the drug and not a dilution effect.

It was then discovered that, although the drug solutions were at pH ~ 7.4, and added to a medium also at pH ~ 7.4, there was still a slight change in the pH, observed as a change in medium coloration. In the main, the NSAIDs made the medium slightly acidic. This may have been due to the NSAID itself.

Alternatively it may have occurred due to intracellular acidification caused by increasing the paracellular conductance of the apical cell membrane to H^+, as shown previously with aspirin in isolated *Necturus* antral mucosa (Kivuluoto *et al.*, 1989). The direct effect of different pH's on the TER was then investigated and pH 4 to 7, and pH 10 greatly reduced the TER, with only pH 8 giving a TER similar to control. Similarly, in Caco-2 (Parisi *et al.*, 1993) and Caco-2BBe monolayers (Menconi *et al.*, 1997), and rat jejunum (Capurro and Parisi, 1992), acidification of the culture medium to pH <7.4 resulted in an increase in paracellular permeability. The effect of pH on the paracellular pathway (i.e. TER) was shown to be via an effect on tight junctions since these were dilated and appeared to contain less of the tight junction-associated protein ZO-1 (Menconi *et al.*, 1997), which is known to diffuse away from the cell periphery in response to a decrease in cellular pH (Li and Poznanasky, 1990). In contrast to the present findings, however, another study showed that T84 and other epithelial cell monolayers, gave an increased TER at pH <4.5 (Chan *et al.*, 1989). This, therefore, indicated that resistance to acid as a general phenomenon of epithelial layers. This is reflected *in vivo*, where the gastric mucosal barrier withstands a luminal pH <1 via acid-induced reduction in the paracellular shunt pathway (Powell, 1981). Other factors which could maintain normal epithelial integrity in the face of acidic challenges include Na^+/H^+ -exchangers in the apical membrane, membrane proton permeability and/or intracellular buffering (Chan *et al.*, 1989). Nevertheless, in order to avoid a possible pH effect, a culture medium buffered with HEPES was used for subsequent experiments in this chapter.

5.9.2 Effect of NSAIDs on Intestinal Cells

Using the HEPES-buffered medium a series of experiments were performed with the NSAIDs indomethacin, aspirin and naproxen, and the prostaglandin

analogue misoprostol. Indomethacin decreased the TER in a concentration-dependent manner within 5 minutes. With naproxen and aspirin, only concentrations greater than 5mM and 10mM respectively, reduced the TER. This would indicate that the potency range of the drugs, that is, the drug which required the least concentration to significantly reduce the TER, was indomethacin > naproxen > aspirin.

In addition, the effect of naproxen on paracellular permeability was assessed using the marker ^{51}CrEDTA. Like [^{14}C]mannitol, ^{51}CrEDTA is a very hydrophilic marker which does not partition into cell membranes and its transport across the epithelial layer takes place around the cells through the tight junction-mediated paracellular pathway. Although the use of [^{14}C]mannitol is more conventional in cellular monolayers (Madara and Dharmsathaphorn, 1985; and Cogburn *et al.*, 1991), ^{51}CrEDTA use is well established for *in vivo* measurements of intestinal permeability (Bjarnason *et al.*, 1995). Also, it has been suggested that due to the smaller molecular size of mannitol (M_w 182), it may even cross the epithelial barrier via the transcellular pathway, whereas ^{51}CrEDTA is a larger probe (M_w 358) so can only cross via the paracellular pathway (Menzies, 1983). The present experiments demonstrated that as the TER decreased over time on treatment of T84 cells with naproxen, the permeability to ^{51}CrEDTA concomitantly increased. This would, therefore, validate the assumption that the decrease in the TER seen with the NSAIDs, is caused by a loosening of intercellular tight junctions. Indeed, increased permeation of ^{51}CrEDTA via the paracellular route has been demonstrated when NSAIDs have been given *in vivo* to human volunteers (Bjarnason *et al.*, 1991) and to experimental animals (Davies *et al.*, 1994).

To date, there is no other documented study of the effect of NSAIDs on the TER with a view to investigating their effects on intestinal permeability. Effects of these compounds on cell lines have been studied for other purposes; the most relevant work will be outlined here.

Damage to Caco-2 monolayers has been demonstrated by subjecting them to 0.5mM indomethacin (Tang *et al.*, 1993). There was a significant reduction in the TER after 1 hour incubation with indomethacin, which was even greater after 12 hours. EM studies revealed that indomethacin induced gross intracellular damage (characterised by granulation), plus damage and/or reduction in the number of microvilli. Indomethacin also significantly increased the flux across the monolayer of [^{3}H]methoxyinulin (which passes through the paracellular pathway), but not [^{14}C]testosterone (which undergoes transcellular transport). Therefore, it can be seen that indomethacin increases paracellular permeability and induces damage in intestinal cells. It is possible *in vivo* that the former will lead to the latter effect.

Although the present studies also showed a reduction in the TER, that seen by Tang *et al.* (1993) was not as profound. In the former studies, the maximum TER decrease with 0.5mM indomethacin was seen within 5 minutes (to 7% of control), whereas with Tang *et al.* (1993) the same concentration of indomethacin gave a maximum reduction in the TER (to only ~50% of control) after 12 hours. Such a discrepancy may have arisen due to the use of different cell lines. Perhaps T84 cells, as used in the present studies, are more sensitive to NSAID effect than Caco-2 cells used by Tang *et al.* (1993). Indeed, the higher TER of the T84 cells will mean that slight changes in the TER are more readily detected.

Another difference was the means of drug addition. In the present experiments, drug solution was added directly to the well, and if pipetted near to the monolayer itself, may have subjected the monolayer to higher than desired drug concentrations. In the experiments of Tang *et al.* (1993), however, the medium bathing the apical chamber was replaced with drug-treated

medium. To validate if the method of drug addition could affect the rate at which the drugs decreased the TER, in one set of experiments for the present work, the medium was replaced with drug-treated medium rather than adding drug directly to the wells. This was found to markedly slow down the rate at which indomethacin and naproxen decreased the TER; it was now observed over hours rather than within seconds.

Another study demonstrated the passage of salicylic acid, the active metabolite of aspirin, across Caco-2 monolayers (Hilgers *et al.*, 1990). This, however, was speculated to be by carrier-mediated transcellular transport rather than paracellular transport since exposure to 40mM salicylic acid for 15 minutes did not alter the TER nor the flux of the [^{3}H]mannitol (Takanaga *et al.*, 1994).

The effect of salicylic acid on resistance has also been investigated in isolated bullfrog gastric mucosa (Kasbekar, 1973). Here, salicylic acid decreased the transmucosal resistance as well as mucosal ATP levels. Furthermore, in accordance with the present hypothesis, it was suggested that uncoupling of oxidative phosphorylation was responsible for the decline in electrical functions of the mucosa. Salicylic acid was also found to induce an increase in paracellular permeability to sucrose.

Several other studies have been carried out in isolated bullfrog gastric mucosa, investigating resistance changes by aspirin. In one study, exposure to

5 and 10mM aspirin for 1 hour or more, reduced mucosal resistance and increased its paracellular permeability to dextran molecules (Flemström and Marsden, 1973). These effects persisted even after removal of aspirin and appeared to be related to aspirin concentration.

In another study, subjecting bullfrog gastric mucosa to 10 or 20mM aspirin caused an initial increase in the transmucosal resistance in the first 35 minutes, followed by a decline to near zero at 70 to 150 minutes (Spenney and Bhown, 1977). Interestingly, mucosal permeability to [^{14}C]mannitol only increased when the resistance had begun to decrease (~ 90 minutes after aspirin addition). However, the possible mechanism for the initial rise in resistance was not speculated upon.

The present work also demonstrated that the prostaglandin analogue misoprostol did not to greatly affect the TER. Although the prostaglandin-mediated protection against epithelial damage seen *in vivo* is also seen in gastric cells cultured *in vitro* (Terano *et al.*, 1982a,b), the present findings indicate that the cytoprotection afforded by prostaglandins is not mediated by alterations in tight junctions. This is consistent with the finding by Weinstein *et al.* (1986), who reported that intragastrically administered misoprostol had no effect on epithelial-cell tight junctions in rat stomach. Indeed, exogenous 16, 16,-dimethyl prostaglandin E_2 (PGE_2) did not prevent the permeability increase caused by aspirin in isolated bull frog gastric mucosa (Spenney and Bhown, 1977).

In contrast, however, results of another study speculated that exogenous PGE_2 did affect intestinal intercellular tight junctions (Krugliak *et al.*, 1990). Such a conclusion was made when PGE_2 was shown to inhibit water absorption in rat intestinal perfusates, possibly by stimulating cyclic 3' 5' -AMP (cAMP).

cAMP increases the TER and, therefore, is thought to be important in the orientation of tight junctional intramembranous strands (Duffey *et al.*, 1981; and Madara, 1988). This, in turn, would affect the paracellular transport of water molecules. However, the study by Krugliak *et al.* (1990), also showed that the NSAIDs indomethacin (2.5 and 5 mM) and aspirin (5 mM) both increased water transport, indicating a 'loosening' of tight junctions. This is in agreement with the present findings.

Lastly, in all the present studies, the final concentration of DMSO as a solvent never exceeded 0.4% and was shown not affect the TER. Even a final concentration of 0.5% has been found to have no effect on tissue resistance or structure (Madara, 1983). Generally, a minimum dilution factor of 1000 is recommended to avoid solvent toxicity (Wilson, 1987). Thus, for TER measurement, DMSO was a highly suitable solvent since any change in the TER was almost certainly caused by the drug and not the solvent.

5.9.3 Effect of Decreased ATP Production on Structure and Function of Tight Junctions

The present work postulates that it is the depletion of mitochondrial ATP production by NSAIDs (by uncoupling and/or inhibition of respiration), which is responsible for the tight junctional changes and thus increase in intestinal permeability. This is compatible with findings that show that proinflammatory oxidants similarly increase paracellular conductance of epithelial monolayers by decreasing ATP levels in the cells (Winter *et al.*, 1990). Moreover, diminished ATP levels are known to evoke profound changes in the organisation of the microfilament-based cytoskeleton (Kellerman and Bogusky, 1992; and Hinshaw *et al.*, 1993). This is substantiated by the *in vitro* finding that filamentous actin assembly from actin monomers

has been shown to be an ATP-requiring biochemical process (Hinshaw *et al.*, 1993). Furthermore, ATP depletion has been shown to rapidly increase transepithelial permeability in T84 monolayers (Mathews *et al.*, 1994) and to lead to a loss of epithelial polarity (Molitoris *et al.*, 1989). Another study demonstrated that the depletion of ATP actually preceded the decrease in TER (Mandel *et al.*, 1993). Such findings indirectly provide evidence for the hypothesis that biochemical changes, such as uncoupling of oxidative phosphorylation, may lead to an increase in intestinal permeability.

Consistent with this is the finding that the respiratory chain inhibitors, cyanide and antimycin A, caused a rapid decrement of ATP levels in MDCK epithelial cells and a decrease in the TER (McCoy *et al.*, 1988; and Mandel *et al.*, 1993). Bacallao *et al.* (1994) extended these observations by reporting that the antimycin A-induced decrease in ATP levels caused significant alterations in the cytoskeleton and tight junctions and that the fall in ATP preceded the drop in the TER. Likewise, in LLC-PK_1 epithelial monolayers, antimycin A depleted ATP levels to <10% of control during which time the TER linearly decreased, and paracellular permeability of [^{3}H]mannitol increased, indicating loss of tight junction integrity, which was then morphologically confirmed (Canfield *et al.*, 1991). When the uncoupler DNP reduced ATP levels, there was a reduced rate of tight junction formation and a rearrangement of tight junction fibrils in HT 29 adenocarcinoma cells (Mückter *et al.*, 1987). The authors stressed, however, that ATP levels required to be reduced severely to ~ 2% of control for an effect, since a drop to 20% was not associated with any tight junctional changes.

In contrast, the uncoupler FCCP was able to induce an abrupt (within 4 minutes) decrease in the TER of MDCK monolayers and open tight junctions, without changes in ATP levels (Li and Poznansky, 1990). Therefore, it appears

that rather than reduced ATP levels being responsible for changes in tight junctional permeability, it is probably due to the breakdown of the proton gradient and the subsequent change in intracellular pH caused by uncoupling of oxidative phosphorylation. Alternatively, it may simply have been that in order for the ATP stores to begin to be depleted, the cells needed to be incubated with FCCP for longer than 4 minutes. This is indicated in the study by Mückter *et al.* (1987), where ATP levels were seen to be reduced after 30 minutes with DNP. Moreover, in the study of Li and Poznansky (1990), a compensatory increase in energy production via glycolysis, may have maintained intracellular ATP concentrations. Indeed, all the former studies mentioned, employed the use of a glucose-free medium and/or 2-deoxyglucose to inhibit glycolysis (Mückter *et al.*, 1987; McCoy *et al.*, 1988; Canfield *et al.*, 1991; Mandel *et al.*, 1993; and Baccallao *et al.*, 1994).

5.9.4 Abnormal Tight Junctional Structure and Function in Disease

Abnormal intestinal permeability caused by perturbation of tight junctions, may be important in the pathogenesis and pathophysiology of a number of diseases including rheumatoid arthritis (Jenkins *et al.*, 1987), indomethacin-associated enteritis (Bjarnason *et al.*, 1987a), and Crohn's disease (Jenkins *et al.*, 1986). Intestinal diseases, where the tight junctional barrier function has been severely altered, are often characterised histologically by small erosions or macroscopic ulcers (Madara, 1989).

During intestinal inflammation, neutrophils (PMN's) migrate out of subepithelial microvasculature, through the paracellular pathway of epithelial cells and accumulate intraluminally to form crypt abscesses. It is thought that it is the adhesion plaques between the PMN's and the epithelial

cells, which form prior to transmigration, that mechanically open the tight junctions (Nash *et al.*, 1987). Simultaneous with their migration, the neutrophils release a plethora of inflammatory mediators, including oxygen metabolites and proteases. These latter agents induce cytoskeletal reorganisation in epithelial cells which, in turn, may also affect tight junctional structure and function (Schneeberger and Lynch, 1992), though this is disputed by Madara (1989). Nevertheless, it is possible that the tight junctional barrier defect might actually enhance the migration of PMN's out of vessels into the intestinal epithelium, where activation of these inflammatory cells would ensue (Hecht *et al.*, 1988).

One such inflammatory mediator is platelet-activating factor (PAF), implicated in ulcerative colitis (Eliakim *et al.*, 1988), Crohn's disease (Kald *et al.*, 1990) and infective colitis (Chaussade *et al.*, 1991). These diseases are all associated with diarrhoea, thought to be caused by PAF-induced increase in paracellular epithelial transport to sodium and water (Travis and Jewell, 1992).

In addition, gamma-interferon (γIFN), a mediator released from lymphoid cells, has been found to substantially reduce intestinal epithelial barrier function by increasing the permeability of tight junctions in T84 monolayers (Madara and Stafford, 1989). Therefore, in chronic inflammatory conditions such as in coeliac sprue and idiopathic inflammatory bowel disease, where the density of lymphoid cells underlying intestinal epithelial cells is particularly prominent (Sleisenger and Fordtran, 1978), γIFN may be partially responsible for the permeability changes seen in the intestine.

Insulin-like growth factor is yet another mediator which highlights a link between increased paracellular permeability and chronic inflammation

(Petruson *et al.*, 1988; and Rom *et al.*, 1988). McRoberts and Riley (1992) have also demonstrated the ability of this factor to mediate an increase in permeability, via the paracellular pathway, in T84 monolayers.

Perturbation of the tight junctional barrier function is a feature of many other disease states with the factors causing these being well documented. These include biologically important factors such as C3a and C5a (Bjork *et al.*, 1985), leukotrienes (Dahlen *et al.*, 1981), histamine (Majno *et al.*, 1969), phorbol esters (Mullin and O'Brien, 1986), neutrophil-derived cathepsin G (Rochat *et al.*, 1988), insulin (McRoberts *et al.*, 1990), and tumour necrosis factor-α (McRoberts and Riley, 1994). All of these agents are associated with either an increase in cell monolayer permeability, and/or with marked cytoskeletal rearrangement.

Likewise, an increase in tight junctional permeability in intestinal epithelial cells has also been observed in the presence of toxins released from microorganisms. Toxin A of *Chlostridium difficile* nearly abolished the TER of T84 monolayers within 6-8 hours, and flux and morphological studies showed that this was due to an enhancement of tight junctional permeability without damage to the integrity of the individual cells (Hecht *et al.*, 1988). When Caco-2 cells were infected with either *Salmonella choleraesuis* or *Salmonella typhimurium*, there was a decrease in TER followed by loss of monolayer integrity, in particular, elongation and denuding of brush border microvilli (Finlay and Falkow, 1990). The toxin of *Vibrio cholera* has also been found to increase tight junctional permeability in intestinal cells (Fasano *et al.*, 1991).

Finally, exposure of epithelial cells to endogenous e.g. hydrogen peroxide (Shasby *et al.*, 1988), or exogenous e.g. NO_2 (Gordon *et al.*, 1986), oxidants, has

been associated with an increase in tight junctional permeability and alteration in tight junctional structure.

Also in Caco-2 cells, the free radical gas nitric oxide (NO), reversibly increased tight junctional permeability by damaging the F-actin in the apical area of the enterocytes, where cell-to-cell contact occurs in the perijunctional ring of actin filaments (Salzman *et al.*, 1995). This in contrast with the purported protective effect of NO at preserving normal intestinal permeability by suppressing the release of other mediators (notably histamine and platelet-activating factor) from mast cells (Payne and Kubes, 1993; and Kanwar *et al.*, 1994). Perhaps the beneficial role of NO reflects its effects on mast cells or other nonenterocytic cells in or adjacent to the gastrointestinal mucosa, absent in cultures of Caco-2 cells.

Salzman *et al.* (1995), also found that NO reduced ATP levels in the Caco-2 cells. However, ultrastructural studies showed that dilatation of the tight junctions by NO was not due to cell death, which may have ultimately resulted from decreased cellular ATP stores. Instead, it appears that it may be a direct effect of ATP on the cytoskeleton which is responsible for the increase in intestinal permeability (see section 5.9.3).

CHAPTER SIX

SUMMARY

6.1 Hypothesis and Original Aims of the Thesis

In the late 50's and early 60's, much investigation concentrated on the biochemical effects of NSAIDs in an attempt to explain better their therapeutic action. It was shown that numerous NSAIDs uncoupled mitochondrial oxidative phosphorylation. Initially this was proposed as the mechanism of action but later it was thought to possibly be involved in the damage caused by these drugs. It then became widely accepted that the therapeutic and side-effects of NSAIDs were caused by inhibition of prostaglandin synthesis. However, the numerous reports demonstrating a lack correlation between prostaglandin inhibition and damage, indicate that this could not be the sole mechanism involved in the damage. Moreover, prostaglandin-independent mechanisms of damage have been suggested including topical irritation, increased intestinal permeability, neutrophil involvement, and *Helicobacter pylori* invasion.

The purpose of this thesis was to accommodate many of the more reliable experimental findings in a new hypothesis for NSAID damage, paying particular attention to the mitochondrial effects as a possible initiator of the intestinal damage. Indeed, mitochondrial dysfunction is a feature of many other disease states (see table 6.1).

The hypothesis investigated for this thesis proposes that, on absorption, the NSAIDs uncouple mitochondrial oxidative phosphorylation. This uncoupling depletes the enterocytes of ATP, which has two effects. Firstly, calcium efflux from mitochondria will trigger a cascade of secondary biochemical events, such as the release of reactive oxygen species (Jain and

Table 6.1 Diseases associated with abnormal mitochondrial function

Disease	Reference
Reye's syndrome	Trost and Lemasters (1996)
Parkinson's	Blandini *et al.* (1998)
Alzheimer's	Sims (1996)
Huntigdon's	Schapira (1997)
Batten	Tanner and Dice (1996)
Diabetes	Gerbitz *et al.* (1996)
Cardiomyopathy	Marin-Garcia *et al.* (1995)

Shohet, 1981), and the activation of proteolytic enzymes (Tuena de Gomes-Puyou *et al.*, 1980). Secondly, ATP synthesis is decreased which results in a disruption of epithelial intercellular junctions whose integrity is controlled by ATP-dependent processes linked to the cytoskeleton (Madara *et al.*, 1987a). This latter effect would lead to an increase in mucosal permeability and the exposure of the mucosa to luminal aggressive factors which, in the small intestine include bile acids, pancreas secretions, ingested food, bacteria and their degradation products, and in the stomach, acid, *Helicobacter pylori* and pepsin. The reduced prostaglandin synthesis, caused by the NSAID inhibition of cyclooxygenase, would prevent repair of the damage, prolonging mucosal exposure to luminal contents. Such exposure will elicit an inflammatory reaction, involving neutrophil chemotaxis and activation, causing indiscriminate damage within the intestinal mucosa followed by blood and protein loss (Bjarnason *et al.*, 1994).

Thus, a multistage framework for the pathogenesis of NSAID-induced GI damage can be envisaged. The initial biochemical stage leading on to the final macroscopic stage, with the transition being the increased in intestinal permeability. However, the idea that the pathogenesis of NSAID-induced GI damage involves several processes is not new. Ivey and Roth (1985) and Szabo (1987) both alluded to there being a series of events leading to the eventual macroscopic damage, with reduced mucosal ATP and prostaglandin levels involved in the early stage of the pathogenesis. Curiously, the same authors did not test their hypotheses at the mitochondrial level.

To test parts of this hypothesis, the effects of NSAIDs on several aspects of mitochondrial function (namely oxidative phosphorylation, electron transport, and ATP generation), was tested. In addition, the effects of these drugs on intestinal permeability was investigated, using intestinal cell lines.

6.2 Summary of the Different Investigations

6.2.1 Uncoupling of Oxidative Phosphorylation

By adding small quantities of isolated rat liver mitochondria to a reaction chamber attached to a Clarke-type oxygen electrode, it was possible to monitor mitochondrial oxygen utilization in the presence of different substrates and drugs. All the NSAIDs and acidic pro-NSAIDs tested, including the active component of nabumetone 6MNA, produced a bell-shape curve when oxygen uptake was expressed a percent of control and plotted against drug concentration. Such a curve is characteristic of "inhibitory uncouplers" (Moreland, 1994). That is, the NSAIDs first uncouple oxidative phosphorylation seen as a stimulation in oxygen uptake, but then at higher concentrations they act like electron transport inhibitors. Both DNP and FCCP also behave like this.

In order for a compound to act as an uncoupler it needs to possess a dissociable group (usually an acid side-chain with a pK_a around 4 to 6), and be lipid soluble (Weinbach and Garbus, 1969). All the conventional NSAIDs tested fulfilled these criteria: possession of an aromatic ring in their structure, a lipophilicity conferring group, and a free acidic group, a carboxylic group or an enolic group in the case of piroxicam (Salguiero-Pagadigorria *et al.*, 1996a). The two exceptions were nabumetone (nonacidic) and paracetamol (pK_a 9). These latter two compounds did not uncouple oxidative phosphorylation and, interestingly, neither of these compounds are associated with gastrointestinal damage in man. Mahmud *et al.* (1996), has demonstrated an

inverse relationship between NSAID pK_a and concentration required for maximum stimulation of respiration.

To further look at the importance of the carboxylic acid side chain in uncoupling, the NSAID flurbiprofen was studied and compared to NO-flurbiprofen, where the side chain had been nitrosylated. As expected, flurbiprofen uncoupled but NO-flurbiprofen did not.

Misoprostol failed to prevent indomethacin-induced uncoupling, indicating that prostaglandins are not involved in the "topical" stage of NSAID-damage. Addition of glucose and citrate also did not affect the uncoupling induced by indomethacin, suggesting that the protective effects of glucose and citrate seen *in vivo* is not a direct protection at the mitochondrial level but an effect related to whole cell metabolism.

The present experiments, therefore, demonstrated that the NSAIDs known to cause GI lesions, also uncoupled mitochondrial oxidative phosphorylation. Although relatively high concentrations of the NSAIDs were required for uncoupling action *in vitro* (0-3.75mM), it is conceivable that such concentrations could be reached within enterocytes during drug absorption, at the therapeutic doses used to treat patients with rheumatoid arthritis (Insel, 1996). Indeed, therapeutic doses of aspirin have been shown to alter mitochondrial structure and function *in vivo* in humans (Lauterburg *et al.*, 1995). However, due to the high concentrations required, it is unlikely that the uncoupling is related to the antiinflammatory or analgesic properties of the NSAIDs (McDougall *et al.*, 1983). It is suggested, therefore, that the uncoupling action of NSAIDs may initiate the associated GI side-effects via a "topical effect".

However, such topical damage seen early on in the pathogenesis of NSAID enteropathy cannot be the sole mechanism since drugs given intravenously, bypassing the local effect, also cause gastric damage (Grossman *et al.*, 1961; and Bugat *et al.*, 1976). Nor too would systemic mediated prostaglandin inhibition alone account for all the deleterious effects of NSAIDs seen at this stage, as suggested previously (see section 1.6.1, chapter 1). Therefore, as already suggested, it seems feasible that the mitochondrial disturbances occur alongside the inhibition of prostaglandin production to produce the lesions.

6.2.2 Inhibition of Respiration

The use of the oxygen electrode, an artificial electron acceptor system and EPR, enabled inhibition of mitochondrial respiration by the drugs indomethacin, aspirin, naproxen and paracetamol, to be localised and characterised. Oxygen uptake studies revealed that all the drugs, including paracetamol, inhibited complex I- and II-mediated mitochondrial respiration in a concentration-dependent manner. The inhibition occurred at higher concentrations than those required to uncouple, a feature seen with most inhibitory uncouplers. But as with the uncoupling, indomethacin was again the most potent at inhibiting mitochondrial respiration.

Using the ferricyanide reductase system, the NSAIDs appeared to preferentially inhibit in the vicinity of complex I. Investigation by EPR demonstrated changes with the NSAIDs in some of the individual components of the respiratory complexes, but no one complex in particular appeared to be targeted. Overall, these inhibition studies indicated that decrease in mitochondrial respiration by the NSAIDs was not localised to one complex or component.

6.2.3 Depletion of ATP Production

Spectroscopic assay of isolated mitochondria incubated with different concentrations of NSAIDs and paracetamol, found that the NSAIDs decreased ATP production concentration-dependently. A depletion in ATP generation following NSAID-induced uncoupling could be responsible for the "local" cell damage seen when NSAIDs are absorbed in the acidic environment of the stomach (Domschke and Domschke, 1984; and Szabo, 1987). The fact that paracetamol did not lower ATP formation suggested two things. Firstly, that the uncoupling action of the drugs is more important in decreasing ATP production than their inhibitory effects. Secondly, that perhaps a longer incubation time than 1 minute was required to cause an adequate depletion. The latter being exemplified by the failure of FCCP, antimycin A or cyanide to give a marked decrease in ATP production.

6.2.4 Affect on Intercellular Tight Junctions

One of the possible consequences of a depletion in ATP production, is an opening of intercellular tight junctions. In cell culture, this is generally assessed as a reduction in the TER of cell monolayers. Therefore, using the intestinal cell lines Caco-2 and T84, an attempt was made to measure their TER and effects of NSAIDs on this using the Ussing chamber. The results from this suggested that cell viability was being challenged and so any further experiments with drugs would give of inaccurate results. Thus, the Millicell electrode was employed, which gave more reproducible results. Here, T84 monolayers were incubated with the NSAIDs indomethacin, aspirin and naproxen, also the prostaglandin misoprostol, and their TER measured. The fact that misoprostol did not reduce TER significantly, would indicate that, like uncoupling, tight junction function is not under prostaglandin control.

In vivo, the loosening of tight junctions would result in the increase in intestinal permeability which in the present *in vitro* experiments was observed as increase in the paracellular transport of ^{51}CrEDTA across T84 monolayers in the presence of naproxen. Thus, overall, there is a strong indication that NSAIDs increase intestinal permeability by loosening tight junctions.

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REVIEW

The Biochemical Basis of Non-Steroidal Anti-Inflammatory Drug-Induced Damage to the Gastrointestinal Tract: A Review and a Hypothesis

Non-steroidal anti-inflammatory drugs (NSAIDs) are amongst the most successful group of drugs ever marketed, which demonstrates their overall efficacy. They are not, however, without side effects (1). The gastrointestinal tract bears the brunt of NSAID-related toxicity (2–5). The gastroduodenal side effects have attracted most attention because of the frequency, severity, and ease of documentation of the damage. Point prevalence studies suggest that 10–30% of patients on NSAIDs have gastroduodenal ulcers (6–10), but the clinical implications of these findings have been played down by suggestions that definitions of ulcer in these studies included a substantial number of large erosions (called endoscopic ulcers; purported to account for up to 50% of 'ulcers' in these studies) of little clinical consequence as opposed to clinically significant ulcers (pre-excising diathesis; implying NSAID-associated rather than NSAID-induced ulcers), which have increased potential to perforate or bleed because of NSAIDs (11). There are, on the other hand, ample experimental data demonstrating NSAID-induced ulcers in all of the intestinal tract of animals and some erosions may be transitory lesions in the development of ulcers in man and hence markers of unacceptable mucosal damage (12).

Because of the ease of documenting the more serious complications of NSAID-induced (or associated) gastroduodenal ulcers—perforation, haemorrhage, or sudden death—there is a tendency to place special reliance on epidemiologic studies that use these features as end-points for 'clinically relevant' side effects of NSAIDs. Even here, however, there is no firm agreement, and estimates vary from NSAIDs being responsible for 30,000 hospital admissions in the UK with an attendant death rate of 10% (13–19) to that of 200–600 premature deaths annually. The fallacy is, however, to let surgeons, epidemiologists, or morticians set the reference for 'clinically relevant outcomes' for conditions that are predominantly managed by physicians. Most gastroenterologic events in patients on NSAIDs are managed by rheumatologists, and when the problems are referred to gastroenterologists, it is mostly because of NSAID-induced dyspepsia and exclusion of malignancy because of iron deficiency rather than because of life-threatening complications. Indeed, all of the gastrointestinal tract may be adversely affected by NSAID ingestion in man.

The role of NSAIDs in the small intestine is clear because there are no confounding variables affecting interpretation of ulcer size and depth, ulcer diathesis, or the possible participation of *Helicobacter pylori*, such as may be the case in the stomach or duodenum (20–26). Sixty per cent of patients on long-term NSAIDs develop small-intestinal inflammation (20, 27–31), with bleeding, protein loss, and, occasionally, strictures (23–25, 32–34). These may lead to management problems (iron deficiency, hypoalbuminaemia) rather than life-threatening events, which places the patients under the care of physicians rather than surgeons, morticians, or epidemiologists.

NSAIDs may also affect the colon, although by comparison this is relatively rare and mostly documented in isolated case reports. NSAID-induced colitis is described following mefenamic acid and may also occur with other NSAIDs (35–40). There is also evidence that NSAIDs are associated with severe fistulous diverticulitis, appendicitis, and relapse of inflammatory bowel disease (41–48).

The mechanism of NSAID damage to the gastrointestinal tract is incompletely understood. Most studies to date have concentrated on altered prostaglandin and leukotriene metabolism or vascular changes as initiating factors (49, 50), and there is considerable information on the importance of bile, refeeding, neutrophils, and bacteria in the development of small-intestinal lesions in experimental animals (51–61). It is now important to summarize and interrelate the more reliable experimental observations in a comprehensive pathogenic framework. The purpose of this review is to provide some evidence for a two-stage pathogenic framework for NSAID-induced damage, which postulates an initial biochemical event at the site of absorption followed by a tissue reaction governed by the luminal aggressive factors, which vary in the stomach, duodenum, and small intestine. In particular, the early biochemical effects of NSAIDs, other than their effects on cyclo-oxygenase, will be reviewed in detail, as there is now, despite some claims to the contrary (62), serious doubt that inhibition of cyclo-oxygenase with reduced mucosal prostaglandins by itself can account for the initiation of the intestinal damage (63–66).

The hypothesis

Fig. 1. outlines the pathogenic framework for NSAID-induced damage to the small intestine. It is suggested that stage one represents specific biochemical alterations initiated by the effect of NSAIDs to uncouple oxidative phos-

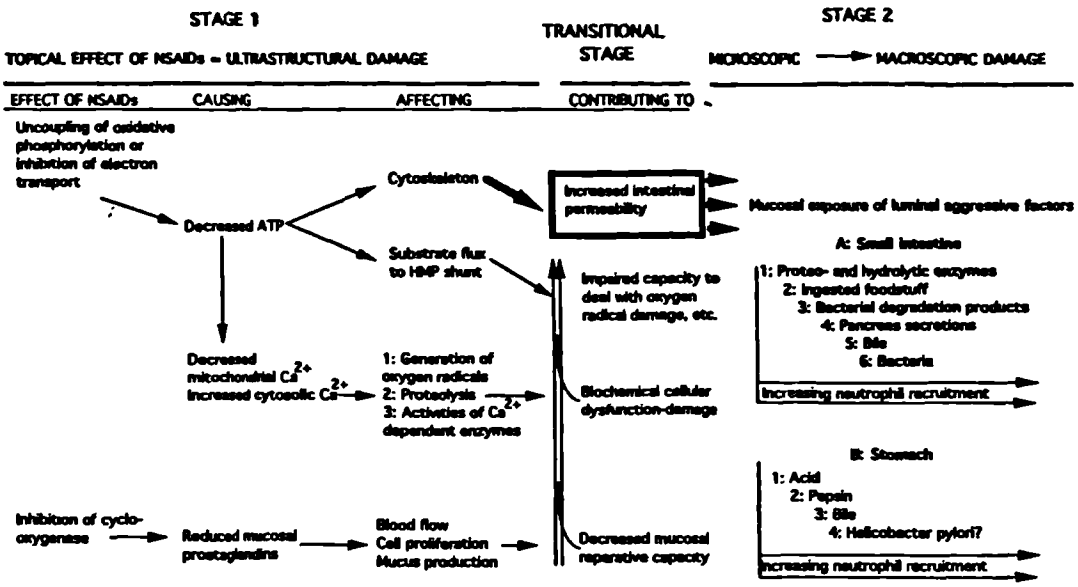

Fig. 1. Outline of the pathogenic framework for non-steroidal anti-inflammatory drug (NSAID)-induced damage to the small intestine and stomach, which differ only in Stage 2. Consult text for details. HMP = hexose monophosphate shunt; ATP = adenosine triphosphate.

phorylation of mitochondria (or inhibition of the electron transport chain), which is most pronounced during drug absorption. The immediate consequence is a reduction in adenosine triphosphate (ATP) production and leakage of Ca^{2+} out of the mitochondria. This leads to i) an increase in cytosolic Ca^{2+} with subsequent Ca^{2+} poisoning (67), ii) damage to mitochondria and an increase in the generation of reactive oxygen species, iii) a disturbance in Na^+/K^+ ratios and cellular osmotic balance (68), iv) a loss of control over intracellular junctions, resulting in increased intestinal permeability (69–75), and v) decreased ATP and increased adenosine diphosphate and monophosphate (ADP-AMP) levels act synergistically to divert glucose 6-phosphate from the hexose monophosphate (HMP) shunt, with loss of reducing equivalents to combat the damage (76).

Increased intestinal permeability is posulated to be the central mechanism of the transition of the effect of NSAIDs from a cellular biochemical (ultrastructural) change to stage two, a tissue reaction (macroscopic). NSAID inhibition of cyclo-oxygenase effectively prevents production of reparative prostaglandins, thus prolonging the permeability changes rather than initiating the damage. The increased intestinal permeability inevitably enables the balance between the interplay of luminal aggressive factors and mucosal defence to be tilted to the advantage of the former, leading to non-specific tissue inflammation. This response may act synergistically with the biochemical damage to further maintain increased intestinal permeability. The first-stage events do not differ among NSAIDs and occur, it is postulated, at any site of absorption whether stomach, duodenum, or the small intestine, which is in turn determined by the drug formulation, size, solubility and pK of the drugs, luminal pH, and so forth. The second-stage tissue response is, however, site-specific. Thus disruption in the gastric mucosal barrier, as proposed by Davenport (77), enables back-diffusion of acid, pepsin, and bile, the main luminal aggressive factors here apart from *Helicobacter pylori*, whereas disruption of the small-bowel barrier exposes the mucosa to digestive enzymes, bile, bacteria, and their degradation products.

We make a clear distinction between the two stages, which is certainly an oversimplification. However, the framework is useful from the investigational point of view and has practical implications for the development of strategies designed to limit and prevent NSAID-induced gastrointestinal damage.

Here follows a detailed account of the biochemical actions of NSAIDs, with emphasis on the small-intestinal lesions, which may be relevant to their adverse action on the whole gastrointestinal tract.

Phase I: the biochemical changes due to NSAIDs

Enterocyte biochemistry. Enterocyte metabolic pathways and their regulation differ from many other cells. Table I shows some enzyme levels in enterocytes which, although only crude indices of metabolic fluxes (78), reflect a high degree of functional differentiation. The intestinal epithelium has a particularly rapid turnover, matched only by

Table I. Distribution of enzymes in the rat small intestine

Name of enzyme	Function	%*
Glucose-6-phosphate dehydrogenase	HMP	88
Phosphogluconate dehydrogenase	HMP	72
Aspartate carbonyl transferase	DNA synthesis	100
Thymidine phosphorylase	DNA synthesis	100
Hexokinase	HMP and glycolysis	71
Peroxidases	Combating oxygen radicals	100
Glutathione disulphite reductase	Combating oxygen radicals	80
Choline esterase	Phospholipid synthesis	100
Lysophospholipase	Prostaglandin synthesis	100

HMP = hexose monophosphate pathway.
* The amount (percentage) of enzyme per wet weight in rat intestine relative to the tissue with the highest concentration, which is shown as 100 (62, 87, 185).

haemopoietic cells of the bone marrow. The high metabolic needs of the enterocytes (79–81) is met by their dual access to high-energy substrates (82) from the lumen and circulation. Luminal glucose (postprandially in concentrations in 10-fold excess of blood) is actively absorbed by Na^+ co-transport (80, 83) and phosphorylated by hexokinase to glucose-6-phosphate. The precise fate of glucose-6-phosphate is uncertain. At least 70% is transported and released as intact glucose into the circulation by the action of glucose-6-phosphatase (78) and specific transporters at the basolateral border (84–86), and about 30% is funnelled into glycolysis and the HMP shunt pathway, depending on the relative activities of phosphofructokinase (PFK) and glucose-6-phosphate dehydrogenase, respectively. The glycolytic capacity of the enterocyte is, however, limited, as the enterocytes have very low levels of phosphofructokinase 2 (87), the main regulator of glycolytic rates (Fig. 2), and most of the glucose-6-phosphate is metabolized via the HMP pathway, in keeping with the very high activities of glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase (Table I).

The HMP shunt has two important roles. First, it converts hexoses to pentoses (mostly ribose) used for nucleic acid synthesis in the rapidly proliferating epithelium. Indeed (Table I), thymidine phosphorylase and aspartate carbamyl transferase activities are also high in comparison with other tissues, reflecting the rapid cell turnover. Second, the HMP pathway provides extramitochondrial reducing equivalents in the form of reduced nicotinamine adenine dinucleotide (NADPH), used in the formation of reduced glutathione (the intestine has high glutathione reductase activity) (88), which is a key compound in limiting the damage of reactive oxygen species (89, 90).

During fasting enterocytes have a major alternative energy supply from glutamine, derived largely from the breakdown of muscle (91). The enterocytes are able to utilize glutamine, unlike many other tissues, because of the high activities of glutamate dehydrogenase, amine oxidase, and transglutaminase (92). Other sources of energy are also readily available, as the enterocyte may use β-hydroxybutyric acid and fatty acids as respiratory fuel (91). The main pathways of ATP production are interregulated, and the dominant pathway is determined by substrate availability (93).

Effect on mitochondria. Most of the work on NSAID-induced toxicity of the small intestine comes from studies in rats, using various doses of indomethacin. There are three important time points of study: i) within 1 h, when there are no detectable light-microscopic changes; ii) at 4–6 h, when inflammatory cell infiltration becomes pronounced; and iii) at 20 h, when macroscopic damage is evident as diffuse, aphthous-like ulceration, predominantly in the middle of the small intestine (94–96).

The earliest evidence of damage following NSAIDs in experimental animals is seen by electron microscopy as swelling, vacuolization, ballooning, and disruption of enterocyte mitochondria (97). This occurs within 1 h of indomethacin administration and is most pronounced in the middle of the small intestine. The picture is seen after uncoupling of oxidative phosphorylation or inhibition of electron transport along the respiratory chain (98). NSAID-induced uncoupling of oxidative phosphorylation (by virtue of their low pK and lipid solubility) has been demonstrated in vitro in gastric tissue with reduced ATP production (96, 99–102). The only other organelle found to be affected at this time was the endoplasmic reticulum (97). At 6 h, and still before macroscopic damage is evident, there are significant changes in the specific activities of marker enzymes of the mitochondria (including citrate synthase, succinate dehydrogenase, cytochrome c oxidase) which are in keeping with the electron microscopy studies (96). Uncoupling of oxidative phosphorylation will increase oxygen consumption and ATPase activities (103, 104). Decreased ATP and accumulation of ADP and AMP result in maximal stimulation of glycolysis. In other cells pyruvate would be converted into acetyl coenzyme A (CoA), but because of the low activities of pyruvate dehydrogenase in enterocytes most of the pyruvate so formed is shuttled into lactate (105). The other effect of maximal stimulation of glycolysis by the above mechanism is to reduce the amount of glucose-6-phosphate available for the hexose monophosphate pathway (76)

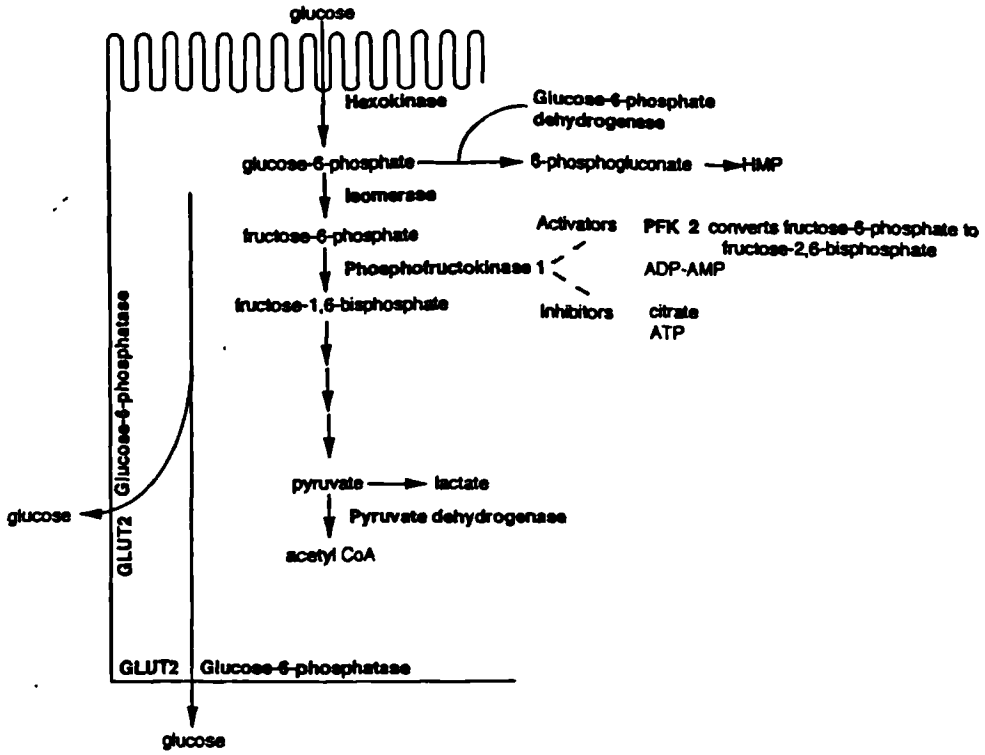

Fig. 2. Fate of glucose in enterocytes. Most of the glucose-6-phosphate is destined for the hexose monophosphate (HMP) shunt or is transported by specific carriers (GLUT 2) at the basolateral borders. A minor portion enters glycolysis because enterocytes lack phosphofructokinase (PFK) 2. What does enter glycolysis is converted to lactate rather than to acetyl coenzyme A (CoA), because pyruvate dehydrogenase activities are low in the intestinal epithelium.

resulting in i) lowered glutathione reductase activity, which is particularly detrimental as NSAIDs inhibit gastric peroxidase (106); both hydroxyl and superoxide radicals are formed as a consequence, with immediate macromolecular damage; *ii*) decreased pentose production for nucleic acid synthesis; and iii) inhibition of NADPH-dependent fatty acid synthesis for maintenance of the brush border.

Lack of ATP because of uncoupling of oxidative phosphorylation or inhibition of the respiratory chain leads to secondary damage by leakage of hydrogen ion into and Ca^{2+} efflux out of mitochondria (107). The biochemical consequences of increased cytosolic Ca^{2+} concentrations include activation of Ca^{2+}-sensitive enzymes, proteases, endonucleases, and phospholipases (107–110). In addition, generation of reactive oxygen species from membrane phospholipids can occur by activation of phospholipase A_2 (111), and depolarization of microtubules will affect the cytoskeleton (112).

Effect on endoplasmic reticulum. NSAIDs cause electron microscopy changes to intestinal endoplasmic reticulum within 1 h of administration, but specific marker enzymes are only abnormal at 24 h, when macroscopic damage is evident (96, 113). NSAIDs inhibit desaturase activities. Desaturases are tightly bound to the endoplasmic reticulum as intrinsic proteins (114–117), requiring oxygen, an electron donor (NADH or NADPH), and cytochrome c reductase. Desaturases are responsible for the synthesis of polyunsaturated fatty acids, which are a component of membrane phospholipids. Inhibition of desaturases causes alterations in the relative proportions of polyunsaturated to saturated fatty acids, resulting in altered membrane fluidity and permeability (118).

Effect on brush border. No structural damage to the brush border membrane is visible by electron microscopy within an hour of indomethacin administration in rats (113), but significant damage is seen by light microscopy at 4–6 h (94, 95) as shortened and blunted villi with polymorphonuclear infiltration of the mucosa. Biochemical marker enzyme studies show significantly lower levels of activity at 6 and 24 h (97) as compared with controls. The biochemical mechanisms of the effect of NSAIDs on the brush border are uncertain, but it is conceivable that the consequence of

reactive oxygen species damage is secondary to Ca^{2+} efflux from mitochondria (119).

NSAIDs increase basolateral membrane Na^+/K^+ ATPase activities involved in the active transport of nutrients (120). Their heightened activity may simply indicate increased metabolic activity and energy requirement of the cell. However, prostaglandin E_2 activates adenylate cyclase, causing increased levels of cAMP, which inhibits Na^+/K^+ATPase (121). Alternatively, NSAID inhibition of prostaglandin generation followed by lowered cAMP and hence increased Na^+/K^+ATPase activities could also explain these findings.

NSAIDs may damage the brush border directly by their action to act as detergents (122, 123), and they may alter the properties of the mucus layer (124), but neither is likely to be important in the initiation of damage, as the histopathologic findings after detergent damage and mucus-altering agents differ markedly from the damage caused by NSAIDs.

Effect on prostaglandin metabolism. In this framework NSAID-induced inhibition does not initiate the intestinal damage. This is clearly a contentious issue, as it has been claimed that intestinal lesions can be produced by including antibodies toward prostaglandins (62). Other data, however, suggest that there is no convincing temporal relationship between inhibition of cyclo-oxygenase and the macroscopic damage (65). There appears to be a lack of relationship between the severity of injury and the reduced mucosal prostaglandins, and a 95% inhibition of prostaglandin synthesis can be achieved without apparent damage (63, 64).

There are at least two forms of cyclo-oxygenase. Cyclo-oxygenase 1 is constitutively expressed in most tissues, where it plays a regulatory role in tissue physiology, whereas cyclo-oxygenase 2 is inducible (not normally present in the intestine) and found mainly at inflammatory sites, playing a part of the inflammatory cascade. NSAIDs have different affinities for the two cyclo-oxygenases, but there is no convincing evidence that NSAIDs with a predominant effect on cyclo-oxygenase 1 (such as piroxicam) differ in their intestinal toxicity from those that are more cyclo-oxygenase-2-specific (such as diclofenac, naproxen). Consideration of the normal physiologic role of prostaglandins (125), namely regulation of blood flow, stimulation of mucus production and facilitation of cell division (126–131), suggests that they are predominantly involved in the regulation of a tissue response. This is difficult to reconcile with their purported role in the initiation of damage. Prostaglandin synthesis is governed by substrate availability and cyclo-oxygenase activity. NSAIDs (and bile acids) may perturb membranes, providing cyclo-oxygenase with its substrate; but this is most likely an indirect action in response to the severe cellular metabolic damage. The cellular action of prostaglandins, however, may be beneficial to the damaged cell. Prostaglandins aggregate with cytosolic-free calcium and may thereby limit cellular damage. Prostaglandins may induce or enhance the radical scavenger system by the induction of superoxide dismutase (132).

Finally, the idea that NSAID-induced injury involves inhibition of cyclo-oxygenase with diversion of arachidonic acid into lipo-oxygenase with production of pro-inflammatory mediators is unlikely, as the two enzymes have different subcellular locations (cytosol and endoplasmic reticulum, respectively) (133). Direct measurement in NSAID-exacerbated experimental colitis also failed to demonstrate any significant shunting of arachidonate into the lipo-oxygenase pathway after NSAIDs (134).

There is considerable interest in the possible pathophysiologic role of other inflammatory mediators (thromboxane, platelet-activating factor) and cytokines (interleukins, tumour necrosis factor and so forth) because of their ulcerogenic potential, pro-inflammatory action, and vascular effect (135–138). These compounds are, by and large, physiologic mediators of inflammation, and although they may play an important role in the pathogenesis of NSAID-induced damage, the evidence is that this occurs as a part of the normal inflammatory cascade. To implicate the lack of prostaglandins in the late biochemical events of NSAID-induced damage is in keeping with otherwise poorly understood observations that pre-treatment with prostaglandins will protect against macroscopic damage from various assaults but not necessarily against microscopic or ultrastructural damage (139–143).

Summary of stage 1. In summary, it is thus proposed that NSAIDs have an initial detrimental biochemical action by uncoupling mitochondrial oxidative phosphorylation and/or inhibition of the electron transport of the respiratory chain. The consequence of uncoupling is lowering of cellular ATP/ADP, which alters intestinal permeability and releases calcium into the cytosol, which in turn causes secondary biochemical damage. NSAID inhibition of cyclo-oxygenase is clearly not the initiator of damage in this framework; rather, it plays a permissive role in the pathogenesis by delaying repair.

Direct support for this hypothesis is presently scant. However, all active NSAIDs have the common physicochemical properties required of an uncoupler—namely, the combination of lipid solubility and a low pK. It has also been shown that when glucose and citrate are co-administered with indomethacin in appropriate concentrations, damage is greatly diminished in the stomach and small intestine (144–146). One possibility is that ATP levels are increased by glycolysis, but it may also be that the simultaneous delivery of glucose and citrate with indomethacin to the enterocyte will counteract some of the secondary metabolic actions of indomethacin. Hence citrate inhibition of glycolysis (effect on PFK) could enable the simultaneously administered glucose to be available for the HMP shunt pathway for production of reactive oxygen species scavengers.

Transitional stage

Role of increased intestinal permeability. The biochemical actions of NSAIDs on the enterocytes causes various kinds of

damage, one of which manifests itself as increased intestinal permeability. The importance of increased intestinal permeability (as measured with molecular weight probes of 300–600 Da) in the pathogenesis of various diseases is circumstantial and rests mainly on the assumption that it is synonymous with increased macromolecular permeation. This is clearly not the case, but there is a significant correlation between increased intestinal permeability and IgA immune complexes in IgA-associated nephropathies, and in the rat there is good correlation between increased intestinal permeability to ^{51}Cr-labelled ethylenediaminetetraacetic acid and ovalbumin and *n*-formyl-methionyl-leucyl-phenylalanine (FMLP; a bacterial neutrophil chemotactic peptide) permeation (147–149). In the context of NSAID-induced enteropathy it is suggested that increased intestinal permeability converts the biochemical damage to a tissue reaction by enabling mucosal exposure of luminal contents that are chemotactic for neutrophils. That this may be the case in man is suggested by the finding that low-grade enteropathies are found after NSAID administration, in chronic renal failure, in treatment with drug therapy for solid tumours, and in human immunodeficiency virus and hypogammaglobulinaemic enteropathy, all of which are preceded by increased intestinal permeability (150–152).

Increased intestinal permeability to NSAIDs occurs within 12 h of ingestion in man. Quantitatively, it relates in a crude way to drug potency to inhibit cyclo-oxygenase and is partially prevented by large doses of synthetic (rioprostol or misoprostol) prostaglandins (20, 21, 146, 153–163). With repeated doses of a NSAID, misoprostol looses its protective effect, suggesting that the biliary component of NSAID damage (not covered by this combination) is sufficient to cause damage (158, 164). If true, this poses a particular problem for the future development of prophylactic measures. Interestingly, NSAID pro-drugs (nabumetone, sulindac, and fenbufen), which are only converted into this active component in the liver after absorption, do not increase intestinal permeability after a week's treatment in man (157, 162). The active component of nabumetone in particular does not appear to be excreted in bile.

Phase II: Non-specific tissue reaction

Luminal aggressors leading to inflammatory changes. Fig. 1 outlines the luminal aggressive factors and how these differ in the stomach and small intestine. It is suggested that the differences in the aggressive factors explain the different tissue responses and hence the macroscopic lesions, depending on site. Here we will concentrate on the small-intestinal aggressive factors and their role in the damage.

There is no doubt about the importance of bile and refeeding of animals after NSAID administration in the pathogenesis of the small-intestinal inflammation caused by NSAIDs (52, 54, 55, 60, 165). The problem has been to explain how they are interconnected and how they may explain the predominant middle and distal small-intestinal location of the damage. The importance of bile is suggested by the finding that bile duct ligation greatly reduces the prevalence of the lesions (54). The luminal concentration of bile acids increases distally as food is absorbed, which may perturb damaged enterocyte membranes and thus act synergistically with the biochemical damage to increase intestinal permeability. Alternatively, most NSAIDs are conjugated and secreted to a significant extent (20–60% of an intravenously administered dose) in bile, which could in practice lead to the selective delivery of large concentrations of NSAIDs to the distal small intestine, where bacterial deconjugation is significant, releasing free and active NSAID (52, 165).

Refeeding animals after the NSAID dose clearly enhances the macroscopic damage. This has usually been taken as evidence of an environmental agent or bacteria playing a pathogenic role in the damage (60). Alternatively, it may simply be that ingested food causes bile secretion with the above consequences.

The role of bacteria has been inferred by the almost universal findings that very few macroscopic lesions are found in the germ-free animal and that pre-treatment with antimicrobials reduces the damage (51–53, 56–58). Curiously, treatment with the same antimicrobials does not facilitate healing in animals (166). But how do the bacteria bring about macroscopic damage?

Knowledge of neutrophil function and pathophysiology may be the key for the understanding of various available experimental results and the sequence of events. Neutrophils only migrate as a co-ordinated response to specific neutrophil chemoattractants (167–171). The mechanisms of neutrophil migration are complex and not fully understood but include adherence to endothelium, which involves expression of endothelial and/or neutrophil receptors (in response to various inflammatory mediators: leukotrienes, tumour necrosis factor, and so forth), aggregation, budding, directed locomotion, and so forth) (172–174). Most importantly, there is an absolute requirement for a neutrophil chemotactic gradient, as without it the neutrophil has no guided locomotion (169, 173, 174), a problem faced by proponents of a vascular/neutrophil-based initiation of NSAID-induced intestinal damage (50, 126, 175, 176). Neutrophil contact with a chemoattractant triggers a metabolic event known as a respiratory burst, which initially involves activation of neutrophil membrane-bound nicotinamide adenine dinucleotide phosphate oxidase with production of superoxide anions (O_2^-) (177–180). Many important reactive oxygen intermediates are produced from the superoxide anion by various catalysts, including hydrogen peroxide, hypochlorous acid, and hydroxyl radical-like compounds, all of which are highly reactive and exert antimicrobial effects. This is followed by internalization of the chemoattractant with subsequent lysosomal enzyme release into the immediate surroundings, provided the concentration of chemoattractant is sufficient (167–171). The neutrophils have dealt

with the immediate problem and have destroyed the chemoattractant, but at the same time there is inevitable tissue damage in the vicinity of the neutrophils.

Neutrophils appear to be the main effector cells in the macroscopic tissue damage in NSAID-induced enteropathy. In man, metronidazole reduced inflammation in all 13 patients so treated (181), and circumstantial data suggest that the putative microbe is invasive (182). Hence there was a significant correlation between inflammation and blood loss in patients with NSAID enteropathy, which is what would be expected if the chemoattractant was within the mucosa, as opposed to minimal damage if it was luminal (182). Similar studies in Crohn's disease and ulcerative colitis suggested a luminal and mucosal location of the neutrophil chemoattractants, respectively (182). Additionally, when treatment of the inflammation is successful in NSAID enteropathy, with metronidazole (181) or sulphasalazine (183), there is concomitant reduction in intestinal blood loss but no significant change in intestinal permeability. This suggests that these agents are acting in the late stages of the pathogenesis of the enteropathy. Similarly, macroscopic damage is limited in rats when neutrophil recruitment is prevented by induction of neutropenia (with antibodies or methotrexate) or after administration of antibodies to adhesion molecules that interfere with anchoring of neutrophil to endothelium (175, 176). What is controversial is the precise mechanism by which the neutrophils make their way to the mucosa. There are several possibilities. In the above framework neutrophil recruitment is due to luminally derived anaerobic microbes or their products, which are directly or indirectly, via macrophage and T-cell activation, chemotactic for neutrophils. Alternatively, others postulate that NSAIDs damage capillary endothelium directly (as a primary event) with subsequent expression of neutrophil adherence molecules and hence the inflammation (50, 126, 175, 176) but face the problem of the lack of guided neutrophil movement (167–171, 174, 184). Furthermore, it is questionable whether neutrophils recruited in this manner are activated, which is after all a prerequisite for damage. Sorting out the precise sequence of events will require careful co-ordinated sequential studies, which include ultrastructural morphologic assessments along with basic biochemical and immunologic techniques.

Conclusion

A multistage pathogenic framework for NSAID-induced enteropathy is presented, encompassing early specific biochemical alterations followed by a non-specific tissue reaction in response to increased intestinal permeability. The framework puts into perspective some of the experimental findings from several workers which are otherwise difficult to understand and invites further experimentation to confirm or refute the hypothesis. The practical aspects relate to future development and targeting of treatment or prevention of NSAID-related damage to the intestine and the possibility that the second stage may have relevance as a common final pathway in the pathogenesis of other intestinal diseases.

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NONSTEROIDAL ANTIINFLAMMATORY DRUGS AND UNCOUPLIN(OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION

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Objective. **There is a lack of correlation between cyclooxygenase (COX) inhibition and nonsteroidal antiinflammatory drug (NSAID)–induced gastrointestinal (GI) damage; it has been suggested that mucosal damage may be initiated by a "topical" action of NSAIDs involving mitochondrial injury. We evaluated the effect of a range of NSAIDs and related compounds on mitochondrial function and assessed the differences between them in relation to their physicochemical properties.**

Methods. **Stimulation of respiration, as an indicator of mitochondrial uncoupling, was measured in isolated coupled rat liver mitochondrial preparations, using an oxygen electrode.**

Results. **Conventional NSAIDs and acidic prodrugs all had stimulatory effects on mitochondrial respiration at micromolar concentrations (0.02–2.7 μM); higher concentrations were inhibitory. The uncoupling potency was inversely correlated with drug pKa ($r = -0.87$, $P < 0.001$; $n = 12$). Drugs known to have good GI tolerability, including modified flurbiprofen (dimeroflurbiprofen and nitrobutyl-flurbiprofen), nabumetone (a non-acidic prodrug), and non-acidic highly selective COX-2 inhibitors, did not cause uncoupling.**

Conclusion. **The ability to uncouple mitochondrial oxidative phosphorylation is a common characteristic of antiinflammatory agents with an ionizable group. Modification or absence of an ionizable moiety reduces the effect on mitochondria and could lea improved NSAID GI safety.**

Nonsteroidal antiinflammatory drugs (NSA are widely used, with more than 26 million and million annual prescriptions in the UK and USA, re tively. The main concern about NSAID treatment i risk of gastrointestinal (GI) damage. Non-ulcer e scopic gastroduodenal lesions are seen in some 30– of patients receiving regular NSAID treatment, peptic ulceration in 10–30% (1,2). In addition, long- NSAID treatment causes small intestinal inflamm (NSAID enteropathy) (associated with blood and tein loss) in ~65% of patients (3).

The mechanism of this NSAID toxicity i known, but, similar to the mechanism of the d antiinflammatory therapeutic effects (4), is widel lieved to be related to inhibition of cyclooxyg (COX) activity. However, there is substantial evic that COX inhibition is not the only pertinent event, 95% inhibition of COX activity has been ach without apparent morphologic mucosal change (5 mice with a disabled prostaglandin synthase 1 (resulting in the virtual absence of endogenous pr glandin) do not spontaneously develop GI lesions. doxically, these mice have been shown to be equa less sensitive to the damaging effects of indometh compared with controls (6). An alternative hypoth that NSAID-induced GI damage is initiated by a " cal" effect in which mitochondrial energy metabol disrupted (7), but alteration in prostanoid product probably an important cofactor in the developme ulcers. NSAID ingestion results in increased activ specific mitochondrial enzymes in rats and in ir mitochondrial changes seen by electron micros compatible with uncoupling of oxidative phosph tion, before macroscopic damage becomes evider Reduced levels of gastric mucosal ATP have documented following NSAID administration, an

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coupling of oxidative phosphorylation has been demonstrated with some NSAIDs (9–12).

Because of renewed interest in the suggestion that the toxic effect of NSAIDs may be initiated by an uncoupling of mitochondrial oxidative phosphorylation and subsequent disruption of cellular energy metabolism, we have evaluated the potency of a range of NSAIDs to stimulate coupled respiration in mitochondria and related this property to the physicochemical nature of the drugs. We tested the hypothesis that the acidic property of NSAIDs (pKa) influences their uncoupling potency and that modification of the carboxyl group may reduce their toxicity and also examined the effects of highly selective COX-2 inhibitors, which are reported to have an excellent GI tolerability profile (13).

MATERIALS AND METHODS

Preparation of mitochondria. Coupled rat liver mitochondria were obtained from 8-week-old Sprague-Dawley rats, as described by Schneider and Hogeboom (14). The animals were killed by cervical dislocation and the liver rapidly dissected and placed in ice-cold homogenizing solution (75 m*M* sucrose, 225 m*M* mannitol, 10 m*M* MOPS, 1 m*M* EDTA, 5 mg/ml bovine serum albumin [BSA], pH 7.4). The liver was cut finely with scissors into approximately 1 × 1 × 1-cm pieces and washed twice with homogenizing solution to remove excess blood. The resultant mixture was suspended in 50 ml of the same solution and homogenized in a Potter-Elvjehm homogenizer by 6 strokes of a rotating Teflon pestle. The homogenate was then centrifuged at 500*g* for 10 minutes to remove excess blood, nuclei, and cell debris. The supernatant was centrifuged at 11,000*g* for another 10 minutes, after which the mitochondrial pellet was carefully removed and resuspended in 40 ml of homogenizing solution. The last centrifugation step was repeated and the mitochondrial pellet resuspended in 1–2 ml of homogenizing buffer. The isolation procedure takes some 45 minutes, with the homogenate kept at between 0°C and −4°C. The mitochondrial protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL), with BSA as the standard protein.

Measurement of oxygen consumption. Oxygen consumption, P:O ratio, and respiratory control ratios were measured by the method of Chance and Williams (15), using a Clarke-type oxygen electrode (Rank Brothers, Cambridge, UK). The electrode was fitted into a thermostated Plexiglas chamber containing 1 ml of oxygen electrode buffer (150 m*M* sucrose, 10 m*M* KCl, 10 m*M* HEPES, 5 m*M* $MgCl_2$, 1 m*M* KH_2PO_4, pH 7.4). Small amounts (20–50 μl) of mitochondrial preparation, 2 μl succinate (20 m*M*), and 0.1–10 μl of drug (to a final concentration of 0.01–10 μ*M*) were introduced through a small hole in the chamber lid. The experiments were carried out at 30°C with continuous magnetic stirring. Baseline oxygen consumption (in the absence of drug) was monitored for approximately 2 minutes at the start of each experiment. Three to eight experiments were performed with each drug concentration. The P:O ratio with succinate as substrate was 1.6. The respiratory control values ranged from 2 to 5.

Reagents. All substrates, the uncoupler carboxyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), and drugs (salicylic acid, acetylsalicylic acid, flurbiprofen, ibuprofen, diclofenac, naproxen, indomethacin, ketoprofen, sulindac, phenylbutazone, piroxicam, fenbufen, etodolac, and acetaminophen) were obtained from Sigma (Dorset, UK). Nitrosalicylic acid was obtained from Fluka (Buchs, Switzerland). Azapropazone was supplied by Wyeth Pharmaceutical (Maidenhead, UK), and the prodrugs nabumetone and 6-methoxy-2-naphthylacetic acid (6-MNA) were supplied by SmithKline Beecham Pharmaceuticals (Welwyn Garden City, Hertfordshire, UK). Nitrobutyl-flurbiprofen was supplied by Nicox SA (Paris, France) and dimero-flurbiprofen by Alchemia (Monza, Italy). NSAIDs were dissolved in 100% DMSO. The final concentration of DMSO in the chamber never exceeded 5%. FCCP was dissolved in 100% ethanol. Control experiments were performed using solvents only. The stock drug solution concentrations ranged from 0.1 m*M* to 2 m*M*.

RESULTS

Relationship of drug pKa to stimulation of coupled respiration by conventional NSAIDs. All of the acidic NSAIDs investigated (nitrosalicylic acid, salicylic acid, acetylsalicylic acid, diclofenac, naproxen, ibuprofen, indomethacin, ketoprofen, piroxicam, phenylbutazone, and azapropazone) stimulated coupled respiration in isolated mitochondria. Figure 1 shows representative findings with indomethacin and ketoprofen. There was an initial increase in the respiratory rate, which reached a maximum with drug concentrations of 0.02–2.7 μ*M* per mg of mitochondrial protein, followed by progressive inhibition of respiration with higher drug concentrations. Acetaminophen, the non-NSAID analgesic control, inhibited mitochondrial respiration only at concentrations higher than those required with the NSAIDs. The vehicle DMSO had no significant effect on mitochondrial function at the volumes tested.

The pKa of the conventional NSAIDs and their potency to uncouple mitochondrial respiration (defined as drug concentration required for maximum stimulation of respiration) are shown in Table 1. The maximum degree of respiration stimulation was similar among the NSAIDs tested, but the concentration needed for maximum stimulation varied with different NSAIDs and was related to drug pKa. Figure 2 shows that there was a highly significant inverse correlation between the NSAID concentration required for maximum stimulation and drug pKa (Spearman's correlation coefficient [r] = −0.87, $P < 0.001$; n = 12).

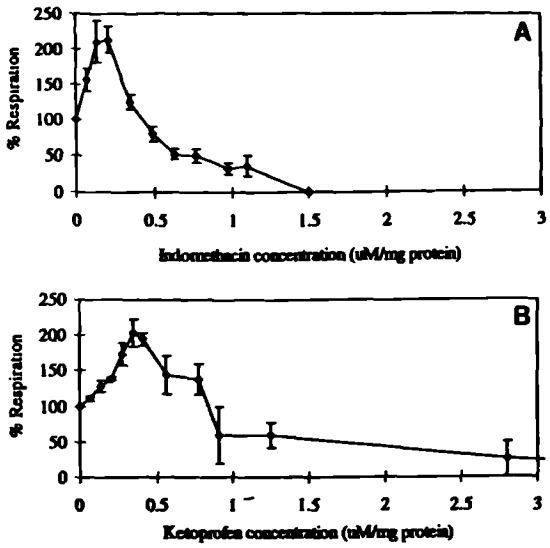

Figure 1. Effects of indomethacin (A) and ketoprofen (B) on coupled mitochondrial respiration. The reaction mixture (at 30°C, pH 7.4) comprised 150 m*M* sucrose, 10 m*M* KCl, 10 m*M* HEPES, 5 m*M* $MgCl_2$, 1 m*M* KH_2PO_4, 20 m*M* succinate (as respiratory substrate), drug concentrations as indicated, and rat liver mitochondrial protein (1.3–4.2 mg/ml). The total volume was 1.0 ml. Values shown are the mean ± SEM of 6 experiments. The rate of oxygen utilization in the Clarke-type oxygen chamber is expressed as a percentage relative to baseline respiration.

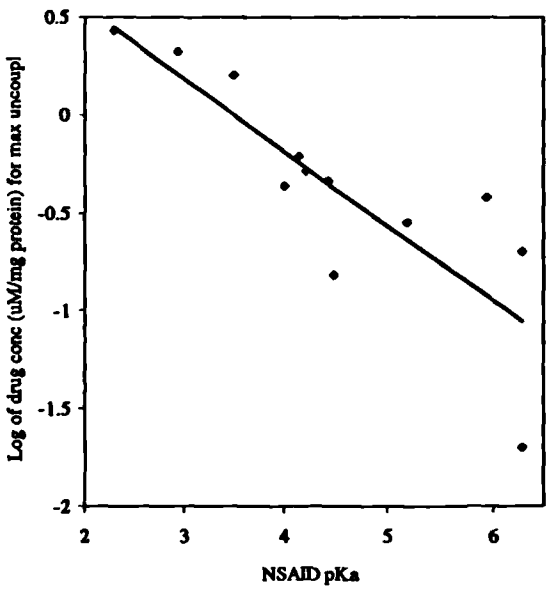

Figure 2. Relationship between the pKa of nonsteroidal antiinflammatory drugs (NSAID) and their potency to uncouple oxidative phosphorylation. There was a highly significant inverse correlation between pKa and concentration (conc) required for maximum stimulation ($r = -0.87$, $P < 0.001$; $n = 12$).

Uncoupling of mitochondrial oxidative phosphorylation by acidic pro-NSAIDs. The findings with acidic pro-NSAIDs (etodolac, fenbufen, and sulindac), were almost identical to the results seen with conventional NSAIDs. Their pKa, concentration required for maximum stimulation, and the maximum degrees of respiratory stimulation are shown in Table 2. In contrast, as shown in Figure 3, the non-acidic pro-NSAID nabumetone (which does not possess a carboxyl or an ionizable group) did not affect mitochondrial respiration

Table 1. Relationship between pKa and uncoupling of mitochondrial oxidative phosphorylation, for conventional NSAIDs and 6-MNA*

Drug	pKa	Percentage maximum uncoupling	Mean ± SEM concentration required for maximum uncoupling (μ*M*/mg protein)
Nitrosalicylic acid	2.3	205	2.70 ± 1.21
Salicylic acid	2.94	200	2.10 ± 1.23
Acetylsalicylic acid	3.5	200	1.6 + 1.19
Diclofenac	4.0	200	0.43 + 0.22
Naproxen	4.15	210	0 61 + 0 16
Flurbiprofen	4 22	265	0.51 + 0.19
Indomethacin	4 5	230	0.15 ± 0.12
6-MNA	5 0	180	0 46 ± 0.27
Ibuprofen	5 2	250	0 28 ± 0.18
Ketoprofen	5 94	220	0.38 + 0.12
Piroxicam	6 3	215	0.20 ± 0.11
Azapropazone	6 3	210	0 02 + 0 02

* NSAIDs nonsteroidal antiinflammatory drugs, 6-MNA 6-methoxy 2 naphthylacetic acid.

Table 2. Relationship between pKa and uncoupling of mitochondrial oxidative phosphorylation, for pro-NSAID, 6-MNA, and COX-2 inhibitors*

Drug	pKa	Percentage maximum uncoupling	Mean ± SEM concentration required for maximum uncoupling (μM/mg protein)
Etodolac	4 65	180	0.5 ± 0.47
Fenbufen	5.7	170	0 46 ± 0.21
Sulindac	4 7	190	0 26 ± 0.18
Nabumetone	–	100	–
6-MNA	5.0	180	0 46 ± 0.27
Nitrobutyl-flurbiprofen	–	100	–
Dimero-flurbiprofen	–	100	–
SC58125	–	100	–
DuP-697	–	100	–

* COX-2 = cyclooxygenase-2. See Table 1 for other definitions.

whereas its carboxylated active metabolite 6-MNA did stimulate respiration at concentrations comparable with those observed for conventional NSAIDs.

Abolition of mitochondrial uncoupling by modification of the carboxylic moiety. The role of the carboxyl group possessed by most NSAIDs (except azapropazone, phenylbutazone, and the oxicams) in uncoupling was explored further using the flurbiprofen derivatives dimero-flurbiprofen (2 flurbiprofen molecules linked by acid anhydride bond) and nitrobutyl-flurbiprofen (carboxyl group esterified with butyl nitric oxide). The chemical structure of these molecules is shown in Figure 4. As seen in Tables 1 and 2, flurbiprofen was a potent uncoupler, but both dimero-flurbiprofen and nitrobutyl-flurbiprofen failed to stimulate mitochondrial respiration.

Lack of effect of COX-2 inhibitors on mitochondrial uncoupling. Two highly selective non-acidic COX-2 inhibitors, SC58125 and DuP-697, had no effects on mitochondrial respiration at concentrations comparable with those used for the conventional NSAIDs (Table 2).

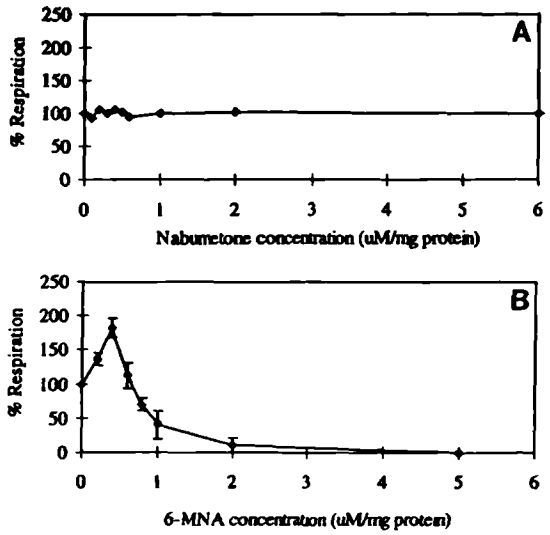

Figure 3. Effects of nabumetone (A) and 6-methoxy-2-naphthylacetic acid (6-MNA) (B) on coupled mitochondrial respiration. The reaction mixture and measurement conditions were similar to those described in Figure 1. Values shown are the mean + SEM of 4 experiments.

DISCUSSION

In this study, commonly prescribed NSAIDs (aspirin, flurbiprofen, diclofenac, indomethacin, naproxen, ibuprofen, piroxicam, and ketoprofen), non-aspirin salicylates, azapropazone, phenylbutazone, and acidic pro-NSAIDs (etodolac, fenbufen, and sulindac), at micromolar concentrations, were found to stimulate coupled respiration in isolated rat liver mitochondria. Acetaminophen did not stimulate mitochondrial respiration. These results and previous data from studies with aspirin (11) and indomethacin (10) indicate a common action of NSAIDs to uncouple mitochondrial oxidative phosphor-

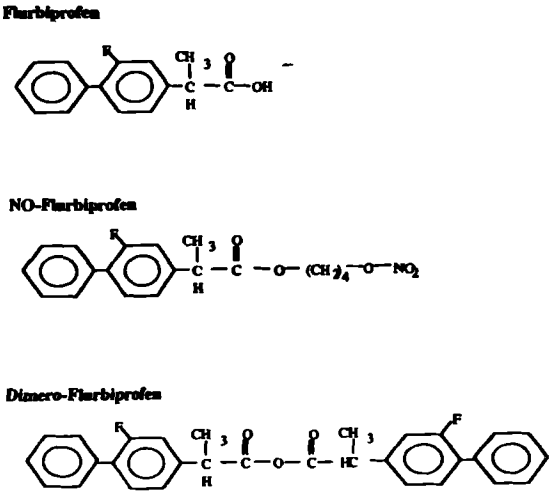

Figure 4. Chemical structures of flurbiprofen and its derivatives. NO-flurbiprofen = nitrobutyl-flurbiprofen.

ylation. All of these drugs are associated with significant GI damage in humans and animals. We expanded on previous studies by assessing a variety of NSAIDs at a range of doses, and we found that their potency to stimulate coupled respiration is significantly related to their pKa. At neutral pH, NSAIDs with low pKa had to be present in higher concentrations in order to uncouple oxidative phosphorylation, presumably because under these circumstances a greater proportion of the drug exists in an ionized state and is thus unable to partition effectively across the inner mitochondrial membrane to translocate protons, compared with a drug whose pKa is close to neutral.

The pathogenesis of NSAID-induced GI injury is probably initiated by a "topical" biochemical damaging action of these drugs (7). It is suggested that a consequential increase in mucosal permeability is transformed into a tissue reaction by mucosal exposure to luminal aggressive factors (7). The ion-trapping hypothesis (16), which postulates that accumulation of NSAID in gastric epithelial cells is dependent largely on interaction of the NSAID pKa and gastric luminal pH (locally), provides a possible explanation for drug absorption (along with molecular size, lipid solubility, dilution, and transit times), but not the mechanism of damage. The common action of acidic NSAIDs on mitochondrial metabolism, suggested previously by Whitehouse (17) and McDougall et al (18), provides a logical explanation of the mechanism of the "topical" phase of damage. Furthermore, butyl–nitric oxide modification of flurbiprofen (19) and other NSAID esterifications that render the drugs non-acidic, and hence ineffective uncouplers (20,21) result in less gastric damage than is seen with the parent compound, when administered on a short-term basis (19,20). In the case of nitrobutyl-flurbiprofen, the nitric oxide released locally during drug absorption may also have a significant role (19).

The relative importance of the "topical" phase in the initiation of the damage, as opposed to systemically mediated inhibition of COX activities, is unknown. The contribution of the former is indirectly supported by enhanced gastric tolerability with changes in drug formulation, such as enteric coating, use of a slow-release preparation, or rectal administration, in short-term endoscopy studies (22). Further support comes from the abrogation of aspirin (pKa 3.5)–induced gastric damage by elimination of acid secretion (23); as a result, aspirin is fully ionized, and hence unavailable for partition across the gastric mucosa. The acidic pro-NSAIDs etodolac, fenbufen, and sulindac all uncouple mitochondrial oxidative phosphorylation and cause significant endoscopic gastroduodenal damage when given on a long-term basis (24,25). However, the non-acidic pro-NSAID nabumetone, which does not uncouple oxidative phosphorylation, causes less GI damage in endoscopic studies (26), and some clinical data suggest a reduced frequency of long-term toxic effects such as peptic ulcers, perforations, and bleeds (27). Since its acidic active metabolite 6-MNA is not excreted in bile, the GI tract is not exposed to uncoupling concentrations of the drug, and this may account for its improved tolerability.

The pathogenesis of NSAID-induced GI disease is clearly a multistep mechanism. It has been suggested that the topical effect of NSAIDs is important in the initiation of this damage and may be due to uncoupling of oxidative phosphorylation. Here we have shown that many commonly used acidic NSAIDs uncouple oxidative phosphorylation, which coincides with their known damaging effect on the GI mucosa, and at neutral pH, their potency to uncouple is inversely related to the pKa of the drug. In the case of flurbiprofen, the uncoupling property was shown to be abolished by nitrobutyl esterification of the parent compound. Furthermore, non-acidic nabumetone and highly selective COX-2 inhibitors, all of which have favorable GI profiles, failed to uncouple oxidative phosphorylation. These results suggest that rendering NSAIDs. incapable of acting as proton translocators may enhance their GI tolerability.

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Intestinal tolerability of nitroxybutyl-flurbiprofen in rats

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Abstract

***Background*—Nitric oxide derivatives of non-steroidal anti-inflammatory drugs (NSAIDs) are thought to be much less ulcerogenic than their parent compounds.**

***Aim*—To compare the effect and potency of flurbiprofen and nitroxybutyl-flurbiprofen to uncouple mitochondrial oxidative phosphorylation (an early pathogenic event in NSAID enteropathy), increase intestinal permeability (transitional stage), and cause macroscopic small intestinal damage.**

***Methods*—In vitro uncoupling potency was assessed using isolated coupled rat liver mitochondria and in vivo by electron microscopy of rat small intestinal mucosa (two hours after the drugs). A dose-response study with flurbiprofen (single doses of 5, 10, 20, and 40 mg/kg) and equimolar doses of nitroxybutyl-flurbiprofen was performed; assessing their effect on intestinal permeability (at 18–20 hours), with ^{51}Cr EDTA, and the number of pointed (<5 mm) and longitudinal (>5 mm) small intestinal ulcers at 24 hours.**

***Results*—Flurbiprofen, but not nitroxybutyl-flurbiprofen, stimulated coupled respiration in vitro. Both drugs, however, uncoupled in vivo; in the case of nitroxybutyl-flurbiprofen possibly because hydrolysis of its ester bond released free flurbiprofen. Intestinal permeability was uniformly and equally increased with both drugs compared with controls. The number of small intestinal ulcers, pointed and longitudinal, was significantly reduced with nitroxybutyl-flurbiprofen apart from the number of longitudinal ulcers with the highest dose.**

***Conclusions*—These studies show that nitroxybutyl-flurbiprofen is associated with significantly less macroscopic damage in the small intestine than flurbiprofen but was associated with mitochondrial damage in vivo and caused similar increases in permeability of the small intestine, suggesting that its beneficial effect is on the later pathogenic stages of the damage.**

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Keywords: intestinal tolerability, nitroxybutyl-flurbiprofen

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The main concern with the use of non-steroidal anti-inflammatory drugs (NSAIDs) is the frequency and severity of their gastrointestinal side effects,[1] which affect both the stomach and small intestine.[2 3] The development of safer NSAIDs depends on insights into the mechanism by which these drugs cause gastrointestinal toxicity. We and others have suggested that there may be two stages to the damage, a topical (biochemical) and a systemic phase (tissue reaction)[4–6] linked by increased intestinal permeability. The topical phase may be due to NSAID induced uncoupling of mitochondrial oxidative phosphorylation (Somasundaram *et al*, manuscript submitted),[6 7] rather than by inhibition of cyclooxygenase[8] and leads to increased intestinal permeability.[9] Subsequently the tissue reaction is the result of mucosal exposure of luminal aggressive factors; neutrophil recruitment and changes in the microcirculation being important for the development of inflammation and ulcers.[6] An alternative suggestion is that NSAIDs damage the intestinal vascular epithelium directly during drug absorption, with expression of adhesion molecules and activation of neutrophils,[10–13] which cause damage by lysosomal release, generation of reactive oxygen species,[14] and by impairing microcirculatory blood flow. There are data to support both the main hypotheses of damage. Based on the this an attempt has been made to limit the gastrointestinal toxicity of NSAIDs by attaching a nitric oxide moiety to conventional NSAIDs in the hope that the nitric oxide released during drug absorption would counteract some of the above detrimental vascular effects. Initial reports of nitric oxide NSAIDs in animals have been encouraging, showing minimal damage compared with the parent NSAID.[15–17]

The attachment of the nitric oxide group to NSAIDs is achieved by linkage of a nitroxybutyric group.[15 16] This connection modifies the NSAID carboxylic group, which is essential for effective inhibition of cyclooxygenase.[18] It is therefore possible that some of the beneficial effect of nitric oxide-NSAIDs (NO-NSAIDs) might be due to rendering them non-acidic and hence ineffective as proton translocators (Somasundaram *et al*, manuscript submitted).[19] Here we report aspects of the topical phase of damage (in vitro and in vivo effects on mitochondria) and changes in intestinal permeability in relation to the macroscopic damage caused by flurbiprofen compared with nitroxybutyl-flurbiprofen (NO-flurbiprofen).

Methods

ANIMALS

Male pathogen free Sprague-Dawley rats (Charles River), 6–8 weeks old and weighing 200–250 g, were used throughout these studies.

A dose response study was done by administration (without sedation) of flurbiprofen (5, 10, 20, and 40 mg/kg), NO-flurbiprofen (7·5, 15, 30, and 60 mg/kg; which are roughly equimolar to the doses of flurbiprofen, respectively), or vehicle by gastric gavage to six (fasting) rats in each group followed by 1 ml water. However, this comparison may be biased against NO-flurbiprofen as previous studies have shown that flurbiprofen and NO-flurbiprofen have comparable efficacy on a weight to weight basis.[15] The highest dose of flurbiprofen was selected to be a roughly equivalent dose (extrapolated from maximum recommended doses in humans) of 20 mg/kg indomethacin in rats, which is the dose of indomethacin most commonly used to induce intestinal damage.

The drugs were initially dissolved in 10% dimethyl sulphoxide and then diluted so that the final concentration of dimethyl sulphoxide was less than 5%. The dissolved drugs were shielded from exposure to light and used within one hour. Control animals received 1 ml vehicle and 1 ml water. After the gavage animals remained fasting for a further two hours after which they had free access to standard rat food and water. They were again fasted the next night and placed in metabolic cages for the intestinal permeability test after which they were anaesthetised (with Hypnovel-Hypnorm) and underwent laparotomy with removal of the whole of the small intestine. Animals were then killed by terminal anaesthesia and cervical dislocation.

PURITY AND STABILITY OF FLURBIPROFEN AND NO-FLURBIPROFEN

The purity of the solutions of flurbiprofen and NO-flurbiprofen was checked using reversed phase high pressure liquid chromatography (HPLC) with ultraviolet detection (276 nm). A Spectrasystem HPLC system was used (Thermo Separation Products, Stone, Cheshire, UK) with a gradient pump (P4000), autosampler (AS3000), and ultraviolet detector (UV2000). Separation was by reversed gradient elution, using a gradient of acetic acid (0·1 mM, pH 4·27) and acetonitrile (0–20 minutes 40% acetonitrile changing from 20–25 minutes to 60% acetonitrile which is maintained until 35 minutes). A C18 column was used (RP-18 LiChrosorb 5 mm, 250×4·6 mm, Phenomenex, Macclesfield, Cheshire, UK) at room temperature. Samples were dissolved in methanol and 25 µl was injected on to the column. The retention times were: flurbiprofen 23·5 minutes, indomethacin (internal standard) 26·4 minutes, and NO-flurbiprofen 39 minutes.

The stability of the two NSAIDs within the intestine was assessed by incubating (shielded from light) flurbiprofen and NO-flurbiprofen for one hour at 37 C with intestinal mucosal scrapings from male Sprague-Dawley rats that had been fasted overnight.

MITOCHONDRIAL FUNCTION

In vitro uncoupling experiments were done on isolated coupled liver mitochondria [2] The liver was cut into 1 cm^3 pieces and homogenised in a 74 mM sucrose, 225 mM mannitol, 1 mM EDTA, 5 mg/ml BSA solution in 10 mM MOPS-NaOH buffer, pH 7·4, by six strokes of a tight fitting teflon pestle. The homogenate was centrifuged at 500 *g* for 10 minutes and the resulting supernatant recentrifuged at 12000 *g* for 10 minutes. The pellet was resuspended in the isolation medium and centrifuged for 10 minutes at 12000 *g*, to give the resulting mitochondrial enriched pellet used for these experiments. All procedures were carried out at 4°C. Protein concentration was determined using a BCA protein assay kit (Pierce, Illinois, USA).

Oxygen consumption, P/O values, and respiratory control ratios were measured with a Clarke type oxygen electrode (Rank Brothers, Cambridge, UK).[21] The reaction mixture consisted of 150 mM sucrose, 10 mM potassium chloride, 5 mM magnesium chloride, and 1 mM potassium phosphate in 10 mM HEPES-NaOH buffer, pH 7·4. Mitochondrial protein (1–5 mg/ml final concentration), substrates, and drugs were introduced into the chamber (1·0 ml capacity) through a small hole in the lid by syringe. Reactions were carried out at 30°C under magnetic stirring. P/O ratios and respiratory control (using carbonyl cyanide-(trifluoromethoxy) phenylhydrazone (FCCP) as uncoupler) were measured on each of the mitochondrial preparations at the start and end of each series of experiments. P/O ratios with succinate as substrate were 4·0 (SEM 1·6). Respiratory control values ranged from 2 to 5.

The effects of drug addition on coupled mitochondrial respiration over a concentration range of 0·01–5·0 µmol/mg mitochondrial protein was monitored by oxygen uptake for two minutes after drug addition. Results are expressed as a mean (SEM) percentage of control (absence of drug) of the duplicates of 4–10 experiments performed on different days.

All substrates, FCCP, and flurbiprofen were obtained from Sigma (Sigma Chemical Company Ltd, Dorset, UK) and NO-flurbiprofen was provided by Dr P Del Soldato (Nicox, Dublin, Ireland).

For the in vitro studies flurbiprofen and NO-flurbiprofen were solubilised in dimethyl sulphoxide before dilution and the pH adjusted to 7·4. Control experiments used the solvent alone. The final concentrations of dimethyl sulphoxide never exceeded 2%.

The in vivo effects of flurbiprofen and NO-flurbiprofen on mitochondria were assessed by electron microscopy. Groups of four rats each received flurbiprofen (10 mg/kg), NO-flurbiprofen (15 mg/kg), or vehicle after an overnight fast, by gastric gavage. Two hours later an abdominal incision was made under anaesthesia, the stomach opened, and a catheter placed in the second part of the duodenum. The whole of the small intestine was then flushed, avoiding distension, with a 3·0% gluteraldehyde solution in 0·1 M sodium phosphate phosphoric acid buffer, pH 7·3–7·4. One cm length of jejunum (30 cm distal to the ligament of Trietz) was then routinely

processed for electron microscopy and placed in gluteraldehyde for three days. After fixation, samples were washed in 1% osmium tetroxide in 0·1 M sodium phosphate buffer (pH 7·3–7·4) followed by a phosphate buffer wash. Dehydration was achieved by increasing concentrations of acetone (25%, 50%, 70%, 90%, 100%) and infiltration in araldite and acetone mixture (50:50) overnight. Samples were washed (two hours) in neat resin, embedded in araldite, and cut ultrathin with an ultratome (Richart Ultracut-E). The samples were then examined with a Joel 1200 cm electron microscope in transmission mode. All samples had been coded and morphology was assessed by one pathologist unaware of the treatments.

INTESTINAL PERMEABILITY

Gastric intubation (without anaesthesia) was carried out after an overnight fast 18–20 hours after receiving the NSAIDs or vehicle. 51-Chromium labelled ethylenediaminetetra-acetate (^{51}CrEDTA; Amersham International, Amersham, UK; 0·5 ml; 10 mCi) was administered (containing approximately 50 nmol ^{51}CrEDTA), followed by 1 ml water.[22] The animals were placed in individual metabolic cages for five hours for collection of urine. Laparotomy was then performed under anaesthesia and the bladder emptied by puncture. The urine obtained was mixed with that excreted during the five hour test period. Urine was counted along with standards in a Wallac 1284 gamma counter for five minutes, which allowed minimal detectable activity of <0·001% of the administered dose.

MACROSCOPIC DAMAGE

The intestinal mucosa was exposed by a cut through the mesenteric side and laid out on a piece of cork for assessment of macroscopic damage 24 hours after giving the drugs. The assessments were carried out by one person who was unaware of the treatments. An ulcer count was made distinguishing between pointed (<5 mm) and longitudinal (>5 mm) ulcers.

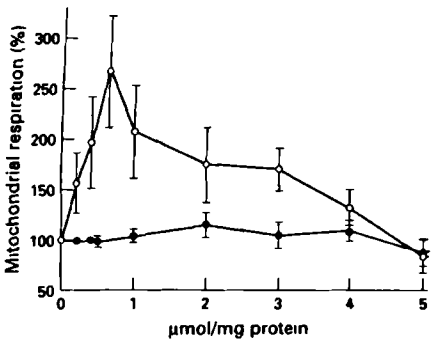

Figure 1 Effect of flurbiprofen (○ and NO-flurbiprofen (● on coupled rat liver mitochondrial respiration in vitro. The rate of oxygen use is expressed as a percentage relative to the baseline respiration. Each data point represents the mean SEM of four to eight experiments

STATISTICAL ANALYSES

The SYSTAT statistic package was used for calculations. Results are expressed as means (SEM). Statistical differences between groups were assessed by the non-parametric Mann-Whitney U test.

Results

MITOCHONDRIAL FUNCTION

Dimethyl sulphoxide alone, at the concentrations and volumes used in the experiments, had no effect on purified mitochondrial oxygen consumption. Figure 1 shows that flurbiprofen stimulates respiration in coupled mitochondria over a concentration range of 0·05 to 4 μmol/mg protein. At concentrations of flurbiprofen above 5 μmol/mg protein respiration is greatly inhibited. NO-flurbiprofen by comparison had no significant effect on mitochondrial respiration over the concentrations tested.

Figure 2 shows representative electron microscopy from animals two hours after receiving vehicle only and the NSAIDs. All four control rats were normal, with mitochondria of uniform size and clearly visible cristae. However, there were identical and equally prominent mitochondrial changes in all the rats receiving flurbiprofen and NO-flurbiprofen. The changes ranged from subtle elongation of mitochondria and swelling to condensation of the matrix due to loss of clarity of the cristae. In some cases there was mitochondrial vacuolisation with disruption of the cristae. The range of abnormalities is compatible and highly suggestive of mild to severe uncoupling of oxidative phosphorylation or inhibition of electron transport,[23] the only other common cause for such changes being exposure to very hyperosmolar solutes.

The endoplasmic reticulum was slightly vacuolated and distended in both groups of animals receiving NSAIDs. The brush border and other subcellular organelles were unaffected by the drugs.

PURITY AND STABILITY OF FLURBIPROFEN AND NO-FLURBIPROFEN

Flurbiprofen and NO-flurbiprofen had distinctly different retention times. Single peaks were obtained with the neat drugs dissolved in dimethyl sulphoxide. However, after incubation with intestinal homogenate for 60 minutes the amount of NO-flurbiprofen was reduced by 50% with a corresponding increase in flurbiprofen, suggesting that the ester linkage of NO-flurbiprofen can be cleaved by intestinal esterases.

INTESTINAL PERMEABILITY

Figure 3 shows that intestinal permeability to ^{51}CrEDTA was significantly increased after each dose of flurbiprofen and NO-flurbiprofen. There was no significant ($p>0·1$ difference between excretion values between groups receiving 5, 10, 20, and 40 mg/kg flurbiprofen or equimolar doses of NO-flurbiprofen

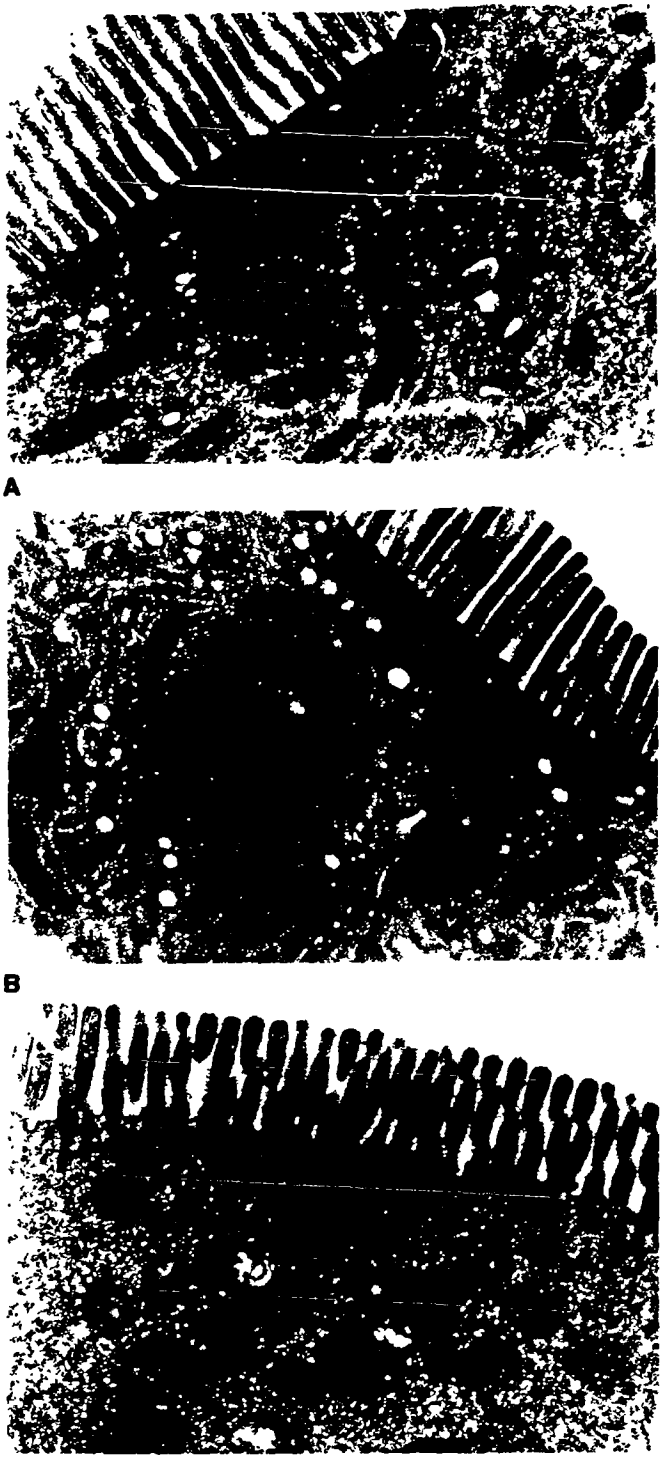

Figure 2: Representative electron microscopic appearances of mucosa of the small intestine two hours after gastric gavage of vehicle, flurbiprofen (10 mg/kg), or NO-flurbiprofen (15 mg/kg). (A) normal appearances of mitochondria after vehicle. The mitochondrial changes after flurbiprofen and NO-flurbiprofen were identical. These ranged from subtle swelling and elongation of mitochondria with loss of clarity of the cristae (B flurbiprofen) to the occasional vacuolisation of mitochondria and disruption f cristae C NO-flurbiprofen) These changes mirror the range of changes in mitochondrial morphology when exposed to increasing concentrations of uncouplers of oxidative phosphorylation. Similar changes are seen with inhibitors of the respiratory chain and very hyperosmotic solutes. Original magnification×14 000

Furthermore, there was no significant difference ($p>0.1$) in the urinary excretion of ^{51}CrEDTA between groups of rats receiving different doses of the drugs.

MACROSCOPIC DAMAGE

Figure 4 shows that the number of pointed ulcers (<5 mm) and longitudinal ulcers (>5 mm) were significantly fewer after NO-flurbiprofen than after each equimolar dose of flurbiprofen except for the highest dose, with which there was no significant difference between the number of longitudinal ulcers between the two drugs.

Discussion

The multistep pathogenic framework for NSAID induced gastrointestinal toxicity proposes that there are distinct and sequential phases of damage. The first is due to the "topical" action of NSAIDs to uncouple oxidative phosphorylation, which leads to increased intestinal permeability (Somasundaram *et al*, manuscript submitted). This may then transform the biochemical phase of damage to an inflammatory reaction, by allowing mucosal exposure of luminal substances, and the development of ulcers mediated either by altered microcirculatory blood flow or by neutrophils.[10–13]

Here we assessed three important pathophysiological aspects of NSAID induced damage to the small intestine at different time points after single doses of flurbiprofen and NO-flurbiprofen. Firstly, we showed that chemical modification of flurbiprofen to NO-flurbiprofen rendered it ineffective as a mitochondrial uncoupler in vitro. Secondly, in vivo the two drugs were equally associated with mitochondrial damage at the electron microscopy level. Thirdly, there was no significant difference between the increased intestinal permeability after the two drugs. Collectively this suggests that the nitric oxide moiety of NO-flurbiprofen does not have a role in altering the early pathogenic events in NSAID enteropathy. A possible reason for the apparent discrepancy between the in vitro and in vivo mitochondrial results is that the ester bond of NO-flurbiprofen may be cleaved by intestinal esterases[24] (present also in abundance in pancreatic secretions) to yield the parent NSAID, which is then able to exert its biochemical effect during drug absorption.

By contrast with the similarities in the early pathophysiological changes seen with flurbiprofen and NO-flurbiprofen, the latter is associated with a significantly improved tolerability of the small intestine as shown in the reduction in number of ulcers, as has been found in the stomach.[15 16] These findings conform to the idea that NO-flurbiprofen releases nitric oxide and thereby causes vasodilatation, increases microcirculatory mucosal blood flow, and prevents neutrophil recruitment,[15 16] all of which are important components in the later stages of NSAID induced gastrointestinal damage.[4 6 11 25] Previous gastrointestinal tolerability studies with NO-flurbiprofen and

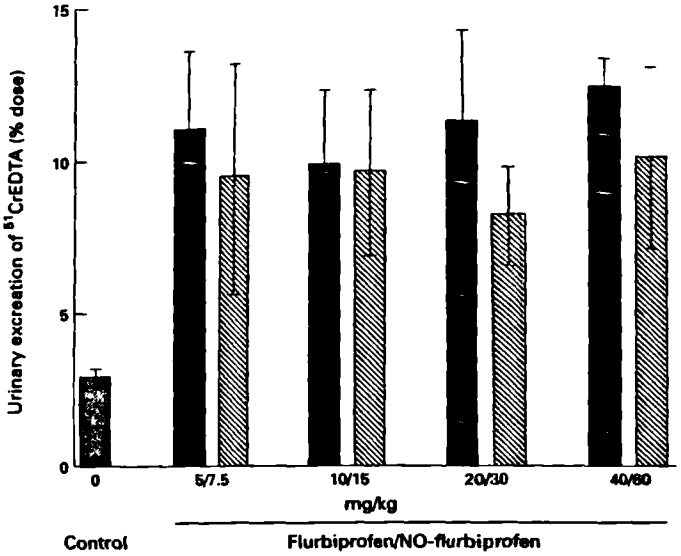

Figure 3: Five hour urinary excretion of ^{51}CrEDTA (mean (SEM)) given 18–20 hours after equimolar doses of flurbiprofen (grey bars) and NO-flurbiprofen (striped bars). Intestinal permeability was significantly increased from baseline at each dose of the NSAID. There was no significant difference in the urinary excretion of ^{51}CrEDTA between the two drugs at any of the doses.

nitrofenac (nitroxybutyl-diclofenac) have shown minimal gastric damage compared with the parent compound when given at doses comparable with those used in the present study.[15 16] Furthermore NO-flurbiprofen given orally to Wister rats (15 mg/kg twice a day) for a week was not associated with any small intestinal pathology whereas all five rats given equimolar doses of flurbiprofen had damage to the small intestine.[15] Similarly, subcutaneous nitrofenac (30 mg/kg twice a day for 3·5 days, and 15 mg/kg twice a day for two weeks) did not cause damage to the small intestine in Wister rats or rabbits, respectively.[16 17] Our results, although not directly comparable because of the different strain of animals used and mode of drug administration, extend these findings and show that at equimolar doses NO-flurbiprofen is associated with significantly less small intestinal ulceration than flurbiprofen. Reduced ulceration along with the findings that it does not seem to affect the early pathogenic events (namely, uncoupling of mitochondrial oxidative phosphorylation leading to the permeability changes) in the damage supports the contention that nitric oxide-NSAID may counteract some of the later vascular effects of NSAIDs. Indirectly this provides some evidence for the importance of the microcirculation in the formation of ulcers as opposed to inflammation which may be predominantly driven by the permeability changes (Somasundaram *et al*, manuscript submitted).[4 26]

Although not directly studied here, it seems possible that the esterification of flurbiprofen which occurs when butyric nitric oxide is attached provides additional protection for the stomach mucosa as this renders the drugs incapable of acting as proton translocators; circumventing the topical phase of damage (Somasundaram *et al*, manuscript submitted). Esterification of other NSAIDs, without introduction of the nitric oxide group, provides compounds with less gastric ulcerogenic action while maintaining in vivo anti-inflammatory activity.[27 28] Because most NSAIDs are relatively ineffective as cyclooxygenase inhibitors without the carboxyl group,[18] it was suggested that in vivo anti-inflammatory activity of esterified NSAIDs was due to generation of the parent compound by intestinal or plasma esterases,[4 27] However, as NO-flurbiprofen is a comparably potent inhibitor of cyclooxygenase to flurbiprofen in vitro[29] neither a difference in cyclooxygenase

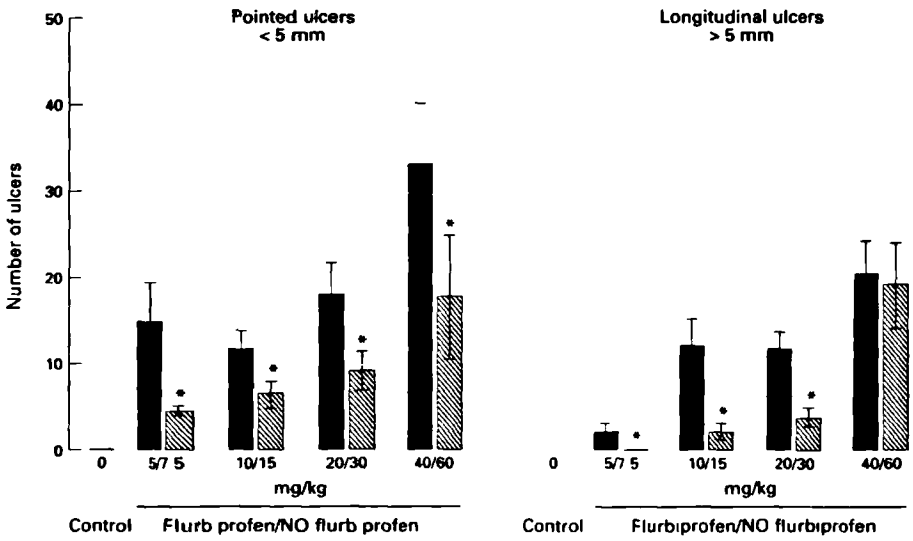

*Figure 4 Number of pointed and longitudinal u ers aft r flurbiprofen grey bars and NO flurbiprofen striped bars) 23 25 hours after administration of hi le or th drug Th number f ulcers t a significant v *p<0 01 less with NO flurbiprofen at ach quimolar se t flurb prof ı . pt ıı th ı ı ber f gıı dıual ı ers with the highest dose*

inhibition or esterification of the NSAID would seem to explain our results. On the contrary there is a differential effect of NO-flurbiprofen and flurbiprofen on the later pathophysiological stages of the damage, as opposed to the early stages, implying an additional beneficial effect of nitric oxide as detailed above. The importance of the nitric oxide group is also evident from other studies, in particular by the finding of Elliott *et al*, who showed that nitrofenac accelerated healing of experimental gastric ulcers in the rat.[30]

In conclusion, orally administered flurbiprofen and NO-flurbiprofen in the rat are equally associated with changes in mitochondrial morphology, that are indicative of uncoupling of oxidative phosphorylation, and increased intestinal permeability. However, the development of ulcers is significantly decreased with NO-flurbiprofen, which is consistent with the suggestion that alterations in the microcirculation may be the driving force in converting the biochemical damage of NSAIDs into ulcers.

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Gut 1997, 41: 344–353

Mitochondrial damage: a possible mechanism of the "topical" phase of NSAID induced injury to the rat intestine

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Abstract

***Background*—The "topical" effect of non-steroidal anti-inflammatory drugs (NSAIDs) seems to be an important cause of NSAID induced gastrointestinal damage.**

***Aim*—To examine the possible mechanism of the "topical" phase of damage in the small intestine.**

***Methods*—Electron microscopy and subcellular organelle marker enzyme studies were done in rat small intestine after oral administration of indomethacin (doses varied between 5 and 30 mg/kg). The effect of conventional and non-acidic NSAIDs on rat liver mitochondrial respiration was measured in vitro in a Clarke-type oxygen electrode.**

***Results*—The subcellular organelle marker enzymes showed mitochondrial and brush border involvement within an hour of indomethacin administration. Electron microscopy showed dose dependent mitochondrial changes following indomethacin administration consistent with uncoupling of oxidative phosphorylation (or inhibition of electron transport) which were indistinguishable from those seen with the uncoupler dinitrophenol. Parenteral indomethacin caused similar changes, but not in rats with ligated bile ducts. A range of NSAIDs, but not paracetamol or non-acidic NSAIDs which have a favourable gastrointestinal tolerability profile, uncoupled oxidative phosphorylation in vitro at micromolar concentrations and inhibited respiration at higher concentrations. In vivo studies with nabumetone and aspirin further suggested that uncoupling or inhibition of electron transport underlies the "topical" phase of NSAID induced damage.**

***Conclusion*—Collectively, these studies suggest that NSAID induced changes in mitochondrial energy production may be an important component of the "topical" phase of damage induction.**

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Keywords: intestinal inflammation, intestinal toxicity, mitochondrial function, drug induced toxicity

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Non-steroidal anti-inflammatory drugs (NSAIDs) are the most prescribed drugs worldwide which attests to their efficacy as analgesics, and anti inflammatory and anti-pyretic agents. The main concern with this group of drugs is the frequency and severity of their gastrointestinal side effects.[1,2]

The pathogenesis of intestinal damage is incompletely understood. It is generally accepted that the early pathogenic events include a "topical" phase in addition to the inhibition of cyclooxygenase, followed by a multistage pathogenic event in which intestinal permeability, luminal contents, neutrophils, and the microcirculation all play a role in the development of inflammation and ulcers.[3–6] The importance of inhibition of cyclooxygenase in the damage induced by NSAIDs has been clearly documented.[3] Nevertheless, it is it is possible to inhibit cyclooxygenase selectively without inducing intestinal damage.[7–9] Furthermore, disruption of the prostaglandin synthase-1 gene coding for cyclooxygenase-1 in mice, with resulting mucosal prostaglandin concentrations of less than 1% of that of controls, is not associated with gastrointestinal pathology.[10] In the stomach changes in drug formulation (enteric coating, rectal administration, etc.)[9,11] and abolishing gastric acid secretion (abrogates aspirin (pKa 3.5) induced damage[12] presumably by limiting the gastric absorption of the drug) greatly increase tolerability of NSAIDs in the short term. This suggests that the "topical" action of NSAIDs may be an important co-factor in the initiation of the damage.[3] The "ion trapping" hypothesis which postulates that accumulation of NSAIDs in intestinal epithelial cells depends largely on the interaction of the acidity of the NSAID (pKa) and luminal pH provides a basis for (along with molecular size, lipid solubility, contact time, etc.) the "topical" action of NSAIDs but not the mechanism.[13,14]

One suggestion is that uncoupling of oxidative phosphorylation may be the biochemical mechanism underlying the "topical" toxicity of NSAIDs.[5,6,15] Uncoupling of oxidative phosphorylation has been documented in vitro with some NSAIDs[3,16,17] and in vivo in the case of aspirin.[18]

The aim of this study was to examine the possible mechanism of the "topical" phase of NSAID induced gastrointestinal damage in the rat by using electron microscopy and subcellular organelle marker enzyme studies

Material and Methods

ANIMALS

Male pathogen-free Sprague Dawley rats (Charles River), 6–8 weeks old weighing 200–250 g were used throughout these studies. For

documentation and confirmation of the ulcer location and frequency, histology, subcellular organelle marker enzyme studies and electron microscopy, animals were fasted overnight before receiving NSAIDs or solvent by gastric gavage via a soft rubber tube (without sedation) at 0800 or 1200 hours. The 0800 hours group of animals remained fasted and were anaesthetised (with Hypnovel-Hypnorm) 1–6 hours later, underwent laparotomy and were killed by cervical dislocation after the procedure. In the case of the animals receiving the drugs at noon, these were re-fed (two hours later) and operated on the next day 20 hours later (with a preceding overnight fast).

For the in vivo studies indomethacin (5, 10, 20 or 30 mg/kg) was dissolved in a bicarbonate buffer (pH 7.4) prior to gastric gavage. The range of indomethacin doses used was similar to that used in most other comparable studies when assessing the acute gastrointestinal toxicity of NSAIDs. These doses are somewhat higher than that required to demonstrate anti-inflammatory activity in carrageenen paw oedema (2.5 mg/kg). Aspirin (100 mg/kg) and nabumetone (600 mg/kg) were initially dissolved in 10% dimethylsulphoxide (DMSO) and diluted to 1 ml just prior to administration (final concentration of DMSO <5%) and was followed by 1 ml water. The doses of aspirin and nabumetone are roughly equivalent to about eight and 21 times the maximum amount given to a 70 kg person with rheumatoid arthritis, respectively (assuming 3–4 g/day for aspirin and 2 g/day nabumetone). The equivalent dose of indomethacin, calculated in the same way, is 2.5-fold (5 mg/kg dose) to 14-fold (30 mg/kg dose) higher. Dinitrophenol (0.4 ml of a 6 mM solution) was dissolved in 10% DMSO and administered at a final concentration of less than 2% DMSO. The same amount of solvent was used in control experiments.

SUBCELLULAR ORGANELLE MARKER ENZYME STUDIES

Groups of eight to 24 rats received indomethacin (30 mg/kg) or solvent by gavage and underwent laparotomy one, six, or 20 hours later. Samples from the duodenum (third part), jejunum (30 cm distal from the ligament of Treitz) and ileum (10 cm proximal to the caecum) were minced finely in a Polytron homogeniser (Kinematica GmbH, Lucern, Switzerland) for 15 seconds at speed setting 1 and disrupted by 15 strokes of a loose fitting (type A) pestle in a Dounce homogeniser in (10% w/v) ice cold 0.25 M sucrose containing disodium EDTA (1 mmol/l, pH 7.4) and ethanol (22 mmol/l). Homogenates were stored at −70°C until assay of enzyme activities.

Organelle specific marker enzymes studies were carried out as described previously[19 20]: alkaline phosphatase (EC 3.1.31), brush border; lactate dehydrogenase (EC 1.1.1.27), cytosol; DNA, nucleus; succinate dehydrogenase (EC 1.3.99.1), inner mitochondrial membrane; citrate synthase (EC 4.1.3.7), mitochondrial matrix (tricarboxylic acid cycle); cytochrome c oxidase 1 9 3 1), mitochondrial matrix (electron transport chain), b-N-acetyl-D-glucosaminidase (EC 3.2.1.30), lysosomes; α-glucosidase (EC 3.2.1.20), endoplasmic reticulum.

Mitochondrial enzymes were assayed after freeze thawing three times to disrupt the mitochondrial membranes.

Protein content was analysed according to Lowry *et al*,[21] and DNA content according to Carter *et al*.[22]

MORPHOLOGY AND ELECTRON MICROSCOPY

A straightforward small intestinal ulcer count was made, following exposure of the mucosa by a longitudinal cut along the mesenteric border, 20 hours after administration of the drugs.

For microscopy an abdominal incision was made under anaesthesia, the stomach was opened and a catheter placed in the first part of the duodenum. The whole of the small intestine was then flushed, avoiding distension, with a 3.0% gluteraldehyde in 0.1 M sodium phosphate buffer pH 7.3–7.4. One centimetre lengths of duodenum (third part), jejunum (30 cm distal to the ligament of Trietz) and ileum (10 cm proximal to the caecum) were then processed: (i) to paraffin wax blocks and 3–4 μm sections were cut and stained with haematoxylin and eosin (six animals each studied as controls and at one, six, or 20 hours after administration of indomethacin 20 mg/kg); or (ii) for electron microscopy and placed in gluteraldehyde for three days (four animals as controls and at one, two, or five hours after indomethacin 20 mg/kg was given). A further four animals in each group were studied by electron microscopy (jejunum only) one hour after instillation in the small intestine of 0.4 ml of a 6 mM solution of dinitrophenol, a potent uncoupler of oxidative phosphorylation, and two hours after administration of 5 and 10 mg/kg indomethacin by gastric gavage to assess a possible dose response relation.

After fixation, samples for electron microscopy were embedded in araldite and ultrathin sections were cut using an Ultratome-Richart Ultracut-E. The samples were then examined with a Joel 1200 cm electron microscope in transmission mode. All experiments were carried out without knowledge of treatment.

IN VITRO MITOCHONDRIAL STUDIES

Animals were fasted overnight. Rat liver mitochondria were isolated and used for these studies as it is exceedingly difficult to obtain high yield, purified coupled mitochondria from intestinal tissue.[23] However, it is generally accepted that there are no major differences between mitochondria isolated from different organs with regard to responses to uncoupling agents or inhibitors of the respiratory chain.[23] After cervical dislocation livers were rapidly removed and placed in ice cold 0 9% saline. The liver was then cut into approximately 1 cm^3 pieces and homogenised in 50 ml of a 74 mM sucrose, 225 mannitol, 1 mM EDTA, 5 mg/ml bovine serum albumin solution in 10 mM MOPS-NaOH buffer, pH 7.4, by six strokes of a tight fitting Teflon pestle The homogenate was centrifuged at 500 *g* for 10

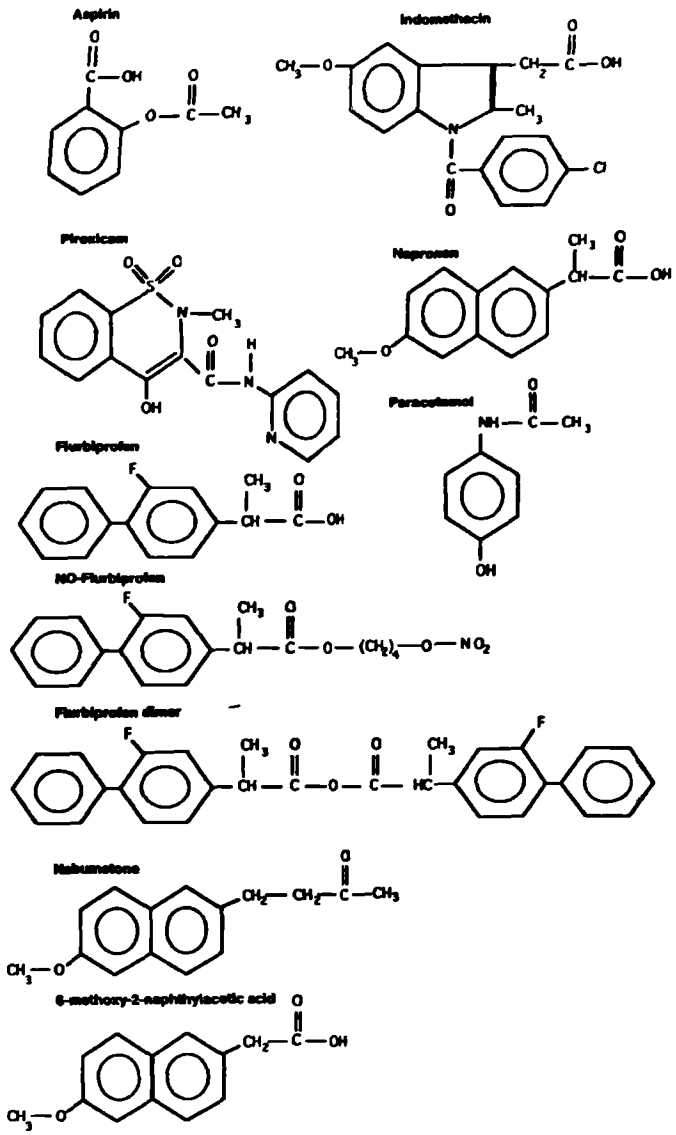

Figure 1: Chemical structure of the NSAIDs and paracetamol used in the study.

minutes and the resulting supernatant re-centrifuged at 12 000 *g* for 10 minutes. The pellet was resuspended in the sucrose solution and centrifuged for 10 minutes at 12 000 *g* to give the resulting mitochondrial enriched pellet used for these experiments. All procedures were performed at 4°C.

Oxygen consumption was measured in a Clarke-type oxygen electrode, as described previously.[24] The reaction mixture consists of 150 mM sucrose, 10 mM potassium chloride, 5 mM magnesium chloride, and 1 mM potassium dihydrogen orthophosphate in 10 mM HEPES-NaOH buffer, pH 7 4. Substrates, inhibitors and drugs were introduced into the chamber (1 ml capacity) by syringe and reactions were carried out at 30 C under magnetic stirring

Electron transport experiments were done with mitoch ndria after addition of 0 1 mM cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) prior to the addition of the drugs

Rotenone and FCCP were dissolved in ethanol whereas the rest were solubilised in 10% DMSO prior to dilution and pH adjusted to 7.4. The solvent was used by itself in the control experiments. The chemical structures of the drugs used in these experiments are shown in fig 1. The conventional NSAIDs studied were indomethacin (pKa 4.5), naproxen (pKa 4.2), aspirin (pKa 3.5), flurbiprofen (pKa 4.2), and piroxicam (pKa 6.3); paracetamol was studied as a non-NSAID analgesic control. Three non-acidic NSAIDs were also studied: nabumetone (and its active acidic metabolite 6-methoxy-2-naphthylacetic acid (6-MNA; pKa 4.5)) and two chemical modifications of flurbiprofen, namely NO-flurbiprofen (fig 1), a nitroxybutylester derivative of flurbiprofen, and a flurbiprofen dimer where a molecule of flurbiprofen is linked to another flurbiprofen molecule via an acid anhydrite bond. Each drug was tested over a range of concentrations and the data presented represent the mean of three to five experiments performed on different days.

Mitochondrial protein was measured by using Pierce's BCA protein assay kit, using bovine serum albumin as standard protein.

BILE DUCT LIGATION EXPERIMENTS

Pharmacokinetics permits three possible routes by which NSAIDs may come into contact with the small intestinal mucosa. Firstly, the "topical" phase following ingestion (during absorption), secondly, a systemic route as the drug enters the vascular compartment and is distributed throughout the body, and, thirdly, following excretion in bile which may re-expose the small intestine to the "topical" phase. In an attempt to discriminate between the "topical" effects of indomethacin on intestinal mitochondrial morphology and the systemically mediated effect, one group of rats underwent bile duct ligation while the other underwent sham operation (all animals were re-fed after the procedure). Twenty four hours later, after an overnight fast, the animals received indomethacin 20 mg/kg intraperitoneally. The animals whose bile ducts had been ligated also received chenodeoxycholic acid by gavage (1 ml of a 10 μmolar solution) the chief constituent of bile acid in the rat. This is an important control as bile plays a pathogenic role in indomethacin induced damage in the rat.[25 26] Electron microscopy (jejunum only) was done two hours later on groups of four animals.

ASPIRIN EXPERIMENTS

Of the NSAIDs studied in the rat, aspirin by gavage (and intraperitoneally)[4] is the only conventional NSAID, apart from nabumetone,[2] that does not cause small intestinal ulcers. Aspirin and nabumetone (6-MNA) are also not excreted in significant amounts in bile following parenteral administration [5] The lack of small intestinal toxicity of aspirin is puzzling One possibility is that the small intestine may not expo ed to significant "topical" concentra

TABLE 1 *Organelle marker enzyme activities in rat jejunum*

Organelle	*Marker*	*Control*	*One hour*	*Six hours*	*Twenty hours*
Nucleus	DNA mg/g tissue)	4 3 (0 8)	4 0 (0 3)	4 0 (0 6)	3 7 (0 5 **
Brush border	Alkaline phosphatase (mU mg DNA)	838 (98)	564 (138)**	432 (85 **	598 (187 **
Cytosol	Lactate dehydrogenase (U mg DNA)	79 (29)	48 (14)	56 (21	83 (16)
Lysosomes	N Acetyl glucosaminidase mU/mg DNA)	135 (41)	125 (44)	151 (40)	155 (39)
Endoplasmic reticulum	α-Glucosidase (U mg DNA)	2 3 (0 2)	2 2 (0 2)	2 7 (0 5)	3 3 (0 7)
Mitochondria	Succinate dehydrogenase mU mg DNA)	102 (18)	168 (61)**	178 (31)**	322 (48)**
	Cytochrome c oxidase (velocity K/min/mg protein)	0 20 (0 04)	0 32 (0.11)**	0 25 (0 07)	0 19 (0 03)
	Citrate synthase (μM/min/mg protein)	1 68 (0 69)	3.05 (0 44)**	3 58 (0 45)**	2 83 (0 52)**

Sprague-Dawley rats (eight to 24 in each group) received indomethacin (30 mg/kg) by gavage and were killed one, six, or 20 hours later

The results show significantly (**p<0 01, Wilcoxon's rank sum test) lower DNA levels at 20 hours possibly because of a reduction in cell numbers as a result of ulceration Alkaline phosphatase activities (brush border marker) are significantly reduced throughout which could represent direct damage by NSAIDs or inactivation due to oxygen reactive metabolites.[11] There are significant increases in the three mitochrondial marker enzyme activities at one hour, albeit transient in the case of cytochrome c oxidase.

tions because of rapid absorption from the gastroduodenal mucosa (and the lack of excretion in bile).[14]

To study this possibility, rats underwent laporatomy under anaesthesia. One group received 100 mg/kg (volume of 1 ml) aspirin directly into the small intestine whereas the other group received the same dose by gastric gavage. Electron macroscopy (jejunum only) was done two hours later on groups of four animals and an ulcer count at 20 hours (six animals in each group).

Results

INDOMETHACIN AND SMALL INTESTINAL LESIONS

Macroscopic, predominantly mid-small intestinal, damage occurred consistently 20 hours after gastric gavage of 20 or 30 mg/kg indomethacin in male Sprague-Dawley rats, as shown previously.[29 30] There were 15–120 ulcers (median 49) in 26 of 30 treated animals. Light microscopy showed no histological inflammatory changes at one hour. At six hours there was a minor polymorphic infiltrate which became intense around the focal erosions and ulcers at 20 hours as described previously in much greater detail by Anthony *et al.*[30]

Indomethacin (5 mg/kg) caused small intestinal ulcers in two of eight animals (numbers 3 and 12) whereas seven (median 22, range 2–56) of 10 animals receiving the 10 mg/kg dose had ulcers.

SUBCELLULAR ORGANELLE ENZYME MARKER STUDIES

After indomethacin (30 mg/kg) ingestion the most noticeable changes in subcellular organelle marker enzyme activity were found in the jejunum (table 1). There was a significant decrease in the activities of the brush border marker (alkaline phosphatase) at one, six, and 20 hours. All three mitochondrial marker enzymes were increased at one hour, persisting throughout in the case of succinate dehydrogenase and citrate synthase. Markers for other subcellular structures did not alter significantly until ulcers were evident, which presumably represents the combination of the biochemical

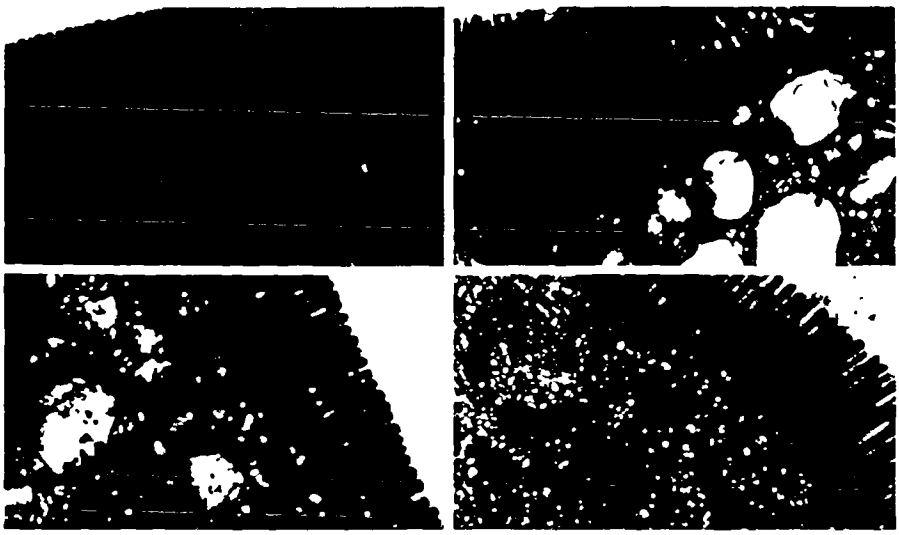

Figure 2 Representative electron transmission micrographs of rat small intestine (jejunum, 30 cm distal to the ligament of Treitz) demonstrating A) normal enterocyte mitochondria, (B vacuolisation of mitochondria one hour after indomethacin administration, (C vacuolisation of mitochondria one hour after the administration of the uncoupler dinitrophenol, and D) normal enterocyte mitochondria one hour after nabumetone administration Note normal brush border appearances Original magnification, ×10 000

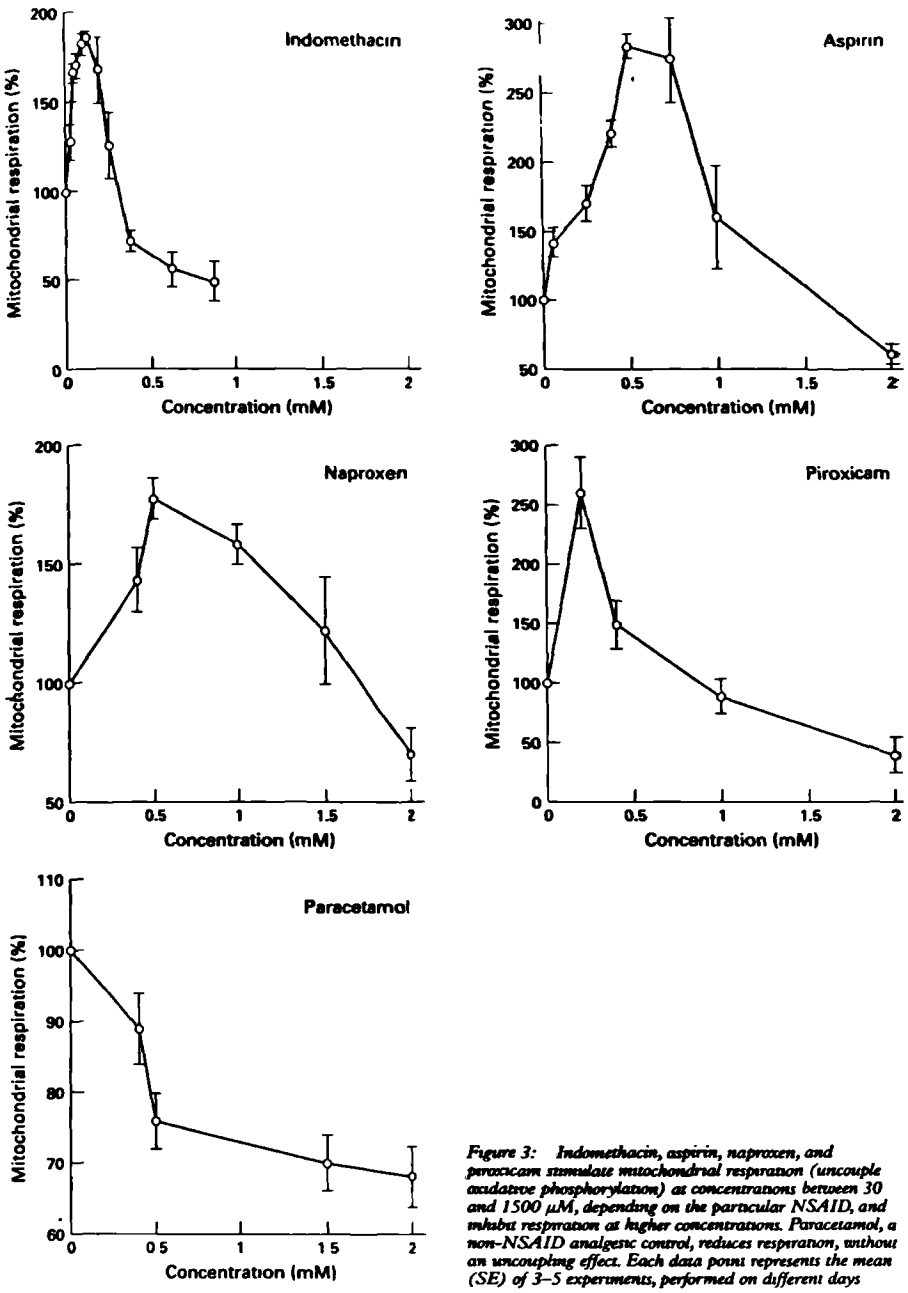

Figure 3: Indomethacin, aspirin, naproxen, and piroxicam stimulate mitochondrial respiration (uncouple oxidative phosphorylation) at concentrations between 30 and 1500 μM, depending on the particular NSAID, and inhibit respiration at higher concentrations. Paracetamol, a non-NSAID analgesic control, reduces respiration, without an uncoupling effect. Each data point represents the mean (SE) of 3–5 experiments, performed on different days

action of indomethacin and the presence and consequence of the damage associated with the acute inflammatory cell infiltrate.

ELECTRON MICROSCOPY

Electron microscopy was carried out in groups of four rats at one, two, or five hours after gavage of 20 mg/kg indomethacin (or vehicle on mucosa from the duodenum, jejunum and ileum. Alterations were found at each site in all the indomethacin treated animals, but were most pronounced in the duodenum and jejunum. Figure 2 shows representative and the most severe changes in mitochondrial morphology in the indomethacin (20 mg/kg) treated rats. These changes were indistinguishable from those found one hour after the administration of the uncoupler dinitrophenol which was associated with the range of mitochondrial morphological changes as described previously in other tissues.[23] Two hours after administration of 5 mg/kg indomethacin there was subtle and patchy swelling and elongation of mitochondria in two rats which increased in severity with the 10 mg/kg indomethacin dose where loss of cristae also became evident, but with only occasional vacuolisation These findings are consistent with

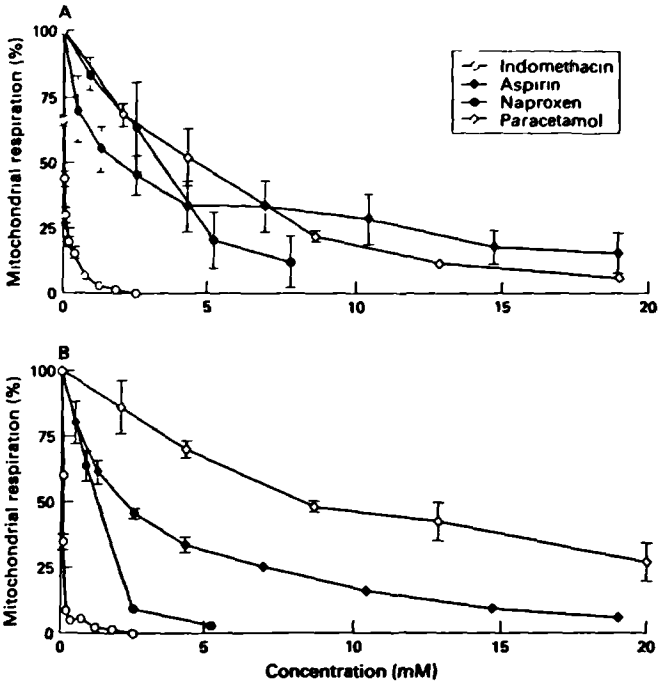

Figure 4: Mitochondrial preparation was as in fig 3, but with the addition of 0.1 μM FCCP, a potent uncoupler, prior to the addition of the drugs. (A) Glutamate/malate and (B) succinate (in the presence of rotenone) were used as electron donors for complexes I and II, respectively, at different concentrations of NSAIDs as shown in the figure. Indomethacin, aspirin, naproxen, and paracetamol all inhibit complexes I and II at different concentrations in a dose dependent manner. Each point represents the mean (SE) of 3–5 separate experiments, performed on different days.

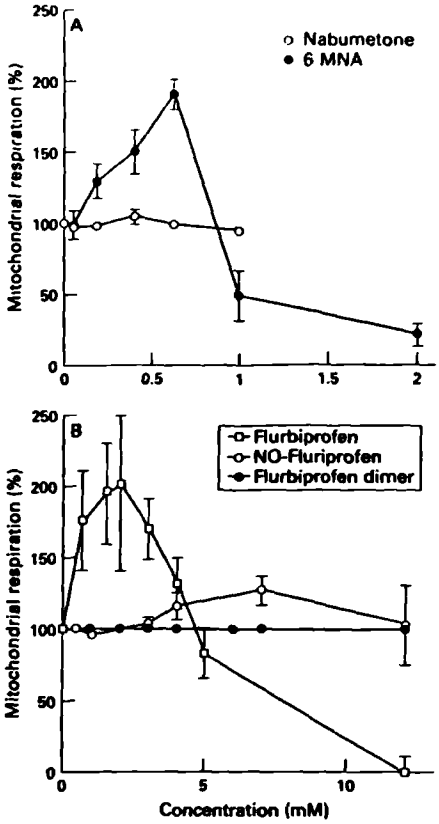

Figure 5: (A) The non-acidic pro-NSAID, nabumetone, had no significant effects on mitochondrial respiration whereas its active component, 6-MNA, stimulated respiration in a similar fashion to the other NSAIDs. (B) Similarly, flurbiprofen stimulated mitochondrial respiration whereas its chemical modification to a nitroxybutyl ester or a flurbiprofen dimer, renders the drugs incapable of uncoupling oxidative phosphorylation.

milder degree of uncoupling or inhibition of electron transport[23] and are within the range of pathology seen with dinitrophenol.

One hour after indomethacin (20 mg/kg) administration, there were also minor degenerative changes (dilatation and some minor vacuolisation) in endoplasmic reticulum, but the brush border and other subcellular organelles appeared normal. There was an impression that the morphological changes became more severe with time.

IN VITRO MITOCHONDRIAL STUDIES

Figure 3 shows that indomethacin, aspirin, naproxen, and piroxicam uncoupled oxidative phosphorylation of isolated rat liver mitochondria in micromolar concentrations whereas paracetamol did not. However, both paracetamol and NSAIDs at higher concentrations inhibited respiration in coupled mitochondria.

The mechanism of this inhibition was studied further. Figure 4 shows that indomethacin, naproxen, aspirin, and paracetamol inhibit both glutamate/malate and succinate (with rotenone) stimulated respiration in uncoupled mitochondria. Using sonicated mitochondria with potassium ferricyanide as an artificial electron acceptor in the presence of appropriate inhibitors (rotenone, antimycin A or cyanide)[21] to define the effects on individual respiratory complexes, all the drugs inhibited electron transfer in complex I and complexes II plus III, in a concentration dependent manner (data not shown).

STUDIES WITH NON-ACIDIC NSAIDs

The effects of nabumetone, a non-acidic pro-NSAID, on in vitro and in vivo uncoupling and its active acidic metabolite 6-MNA in vitro, were studied.

A single 600 mg/kg dose of nabumetone by gastric gavage in eight rats did not cause macroscopic or microscopic small intestinal damage at 20 hours. Furthermore, electron microscopy of the mid-small intestine (four rats) following this dose at one and five hours showed no alterations in mitochondrial morphology (fig 2). Nabumetone also had no effect on mitochondrial respiration in isolated coupled rat liver mitochondria up to a maximum solubility (about 1.0 mM) in 10% DMSO fig 5). The active metabolite of 6-MNA, however, increased respiration by 180 to 200% in the concentration range of 0.3–0.7 mM.

Figure 5 shows that the chemically modified versions of flurbiprofen (nitroxybutyl-flurbiprofen and flurbiprofen dimer), which render them non-acidic and in the case of the former abolish gastric toxicity and reduce small intestinal damage significantly,[1] had no significant effects on mitochondrial respiration.

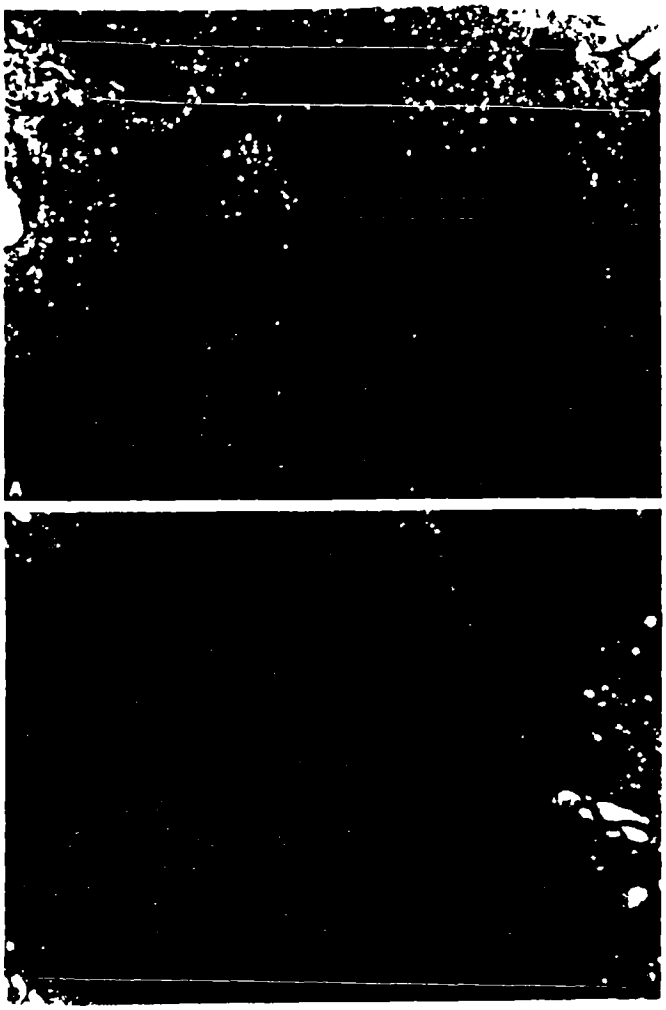

Figure 6: Representative electron transmission micrographs of rat small intestine following intraperitoneal indomethacin (20 mg/kg) in (A) a rat with a ligated bile duct showing normal intestinal mitochondria with discrete cristae and (B) a sham operated animal showing disruption of mitochondrial cristae, vacuolisation and occasional disruption of both the outer and inner mitochondrial membranes. Endoplasmic reticulum is also dilated. Original magnification, × 30 000.

BILE DUCT LIGATION EXPERIMENTS

Sham operated animals with intact bile ducts, receiving indomethacin intraperitoneally, had abnormal jejunal mitochondria (representative picture shown in fig 6) resembling that seen after gavage (as above), whereas rats with ligated bile ducts had normal mitochondrial morphology. Collectively, this shows that the systemically mediated effect of indomethacin, at the doses given, is insufficient to affect small intestinal mitochondrial morphology whereas the biliary excreted component seems sufficient to cause the "topical" effect.

ASPIRIN EXPERIMENTS

Aspirin uncoupled in vitro (fig 3). Animals receiving aspirin by gastric gavage had normal jejunal mitochondrial morphology on electron microscopy whereas those receiving aspirin directly into the small intestine had changes identical to those following indomethacin and dinitrophenol suggestive of uncoupling of oxidative phosphorylation and/or inhibition of electron transport. None of the animals receiving aspirin by gavage had small intestinal ulcers whereas those receiving it directly into the small bowel had severe, extensive and confluent small intestinal ulceration distal to the administration site of aspirin, suggesting that the lack of apparent small intestinal toxicity of orally administered aspirin may be due to lack of the "topical" effect because of rapid absorption.[14]

Discussion

Here, we show directly by a subcellular marker enzyme technique that intestinal mitochondria and brush border are affected within one hour of indomethacin administration. Electron microscopy shows dose dependent mitochondrial changes that are indicative of uncoupling of oxidative phosphorylation or inhibition of electron transport. We expand on the previous in vitro studies of NSAIDs on mitochondrial energy production, show that uncoupling relates to the acidic nature of NSAIDs and that rendering NSAIDs non-acidic and ineffective as proton translocators coincides with their improved gastrointestinal tolerability. Along with the bile duct ligation and aspirin studies, this suggests that the mechanism of the "topical" phase of NSAID damage may involve uncoupling of oxidative phosphorylation and/or inhibition of electron transport.

Although the pathogenic events of NSAID induced gastrointestinal damage are controversial, there is a consensus that there is an important "topical" component of the damage both in the stomach and small intestine[9 33-35] which is independent of NSAID action to inhibit cyclooxygenase.[3 36] The precise nature of the "topical" phase of damage is uncertain. Firstly, one suggestion is that NSAIDs have a detergent-like action which disrupts mucus gel[37] and/or cell membrane integrity.[38 39] The decreased activity of the brush border enzyme alkaline phosphatase is consistent with this suggestion, although no major structural changes were seen on electron microscopy. Secondly, acidic NSAIDs may concentrate in the mucosa ("ion trapping"),[40 41] but how this causes damage is uncertain. The subcellular organelle marker enzyme studies suggest that mitochondria are affected following oral indomethacin. Mitochondrial enzyme activities were all significantly increased, which is, however, an unusual response to damage and not readily explained. One possibility is that substrate access to the membrane bound enzymes is facilitated via increased mitochondrial membrane permeability caused by the effect of indomethacin on mitochondrial energy production Whatever the explanation, the electron microscopy studies showed dose dependent in vivo mitochondrial changes following indomethacin administration which are suggestive of uncoupling and/or inhibition of electron transport. The in vitro studies show that all the acidic NSAIDs uncoupled oxidative phosphorylation at concentrations which are likely to be achieved within intestinal epithelial cells following ingestion of NSAID , whereas

paracetamol, an analgesic without gastrointestinal toxicity, and the non-acidic NSAIDs which have an improved gastrointestinal tolerability profile, did not. These findings expand on earlier studies which demonstrated that some NSAIDs uncoupled oxidative phosphorylation,[41 42] and indirect evidence that suggested that uncoupling of oxidative phosphorylation or inhibition of electron transport may play a role in their intestinal toxicity.[43 44] Furthermore, our studies show that the uncoupling property of NSAIDs reside within their carboxylic or enolic acid groups (along with their lipid solubility characteristics).

The doses of indomethacin used to demonstrate in vivo uncoupling in rat small intestine were 2.5- to 14-fold higher than maximum doses used in a 70 kg human. Administration of these high doses, notwithstanding differences in species susceptibility, is a compromise between the desire to use comparable pharmacological doses as in humans and those required for consistent ulcer formation in the rat. The doses of indomethacin used in this study, although an order of magnitude greater than that required to inhibit cyclooxygenase,[45 46] are, however, within the range conventionally used to investigate the pathogenesis of NSAID induced gastrointestinal toxicity.

The "topical" effect of NSAIDs is most pronounced at the site of drug absorption following ingestion. Absorbed NSAIDs are largely (over 99%) bound to albumin[28] so that an effective concentration for mitochondrial uncoupling may not be reached in other tissues. Certainly, there was no evidence of uncoupling of small intestinal mitochondria when indomethacin was administered parenterally to rats with ligated bile ducts. However, the main objection to the importance of the "topical" phase of damage of NSAIDs comes from the observations that: (i) parenterally administered NSAIDs cause small intestinal damage[47 48]; (ii) oral aspirin does not cause small intestinal damage (which also suggests that inhibition of cyclooxygenase is not the only explanation for the damage). In respect of (i) our studies show that parenteral indomethacin uncouples intestinal mitochondrial oxidative phosphorylation or inhibits electron transport via excretion in bile. Indeed most NSAIDs, apart from the active metabolite of nabumetone, 6-MNA, and aspirin have a significant biliary excretion component[27 49] which exposes both the stomach (reflux of duodenal contents) and the small intestine to concentrations of the drug which may be sufficiently high to uncouple, depending on the doses given. We could not demonstrate small intestinal toxicity following very large doses of nabumetone or an effect to uncouple oxidative phosphorylation in vivo or in vitro which accords with its purported favourable gastrointestinal side effect profile in humans.[2 50 51] However, the active metabolite of nabumetone, 6-MNA, uncoupled mitochondria in vitro, which in keeping with the results of the other acidic NSAIDs. Although oral 6-MNA clearly causes gastric damage (small bowel damage was not looked for), large intravenous doses of 6 MNA cause no intestinal damage, possibly because the intestine is not exposed to intact 6-MNA[17] when administered by this route. Lastly, the nitroxybutyl modification of flurbiprofen or the flurbiprofen dimer renders the drug non-acidic and ineffective as a mechanical proton translocator. Wallace *et al*[52] showed that the former modification diminishes or abolishes the gastric toxicity of the drug. It was suggested that the basis for reduced toxicity was the release of nitrogen oxide from the molecule, but the present studies suggest that it might additionally be due to the modification of the carboxyl group, rendering the molecule inactive as an uncoupler of oxidative phosphorylation. In this context it is interesting that esterification of aspirin is associated with strikingly improved short term gastric tolerability in the rat.[53] Collectively, these findings support an important pathogenic role of the "topical" action of NSAIDs in intestinal damage.

Aspirin is clearly toxic to the gastric mucosa, but it has been particularly difficult to explain why it does not cause small intestinal damage regardless of the route of administration (orally or parenterally). Here, we show that when aspirin is instilled directly into the small intestine it is associated with uncoupling of intestinal mitochondrial oxidative phosphorylation and/or inhibition of electron transport and leads to severe small intestinal ulceration, but not when given by gastric gavage. This suggests that aspirin is so rapidly absorbed by the gastroduodenal mucosa following ingestion[14] and that insufficient concentrations are achieved in the more distal small intestine (along with its lack of excretion in bile) to affect mitochondria and hence it does not exert a "topical" action at this site or cause damage. Indeed the gastric "barrier breaking" effect of aspirin is only seen following ingestion and not after intravenous administration[9 47] of moderate doses. Parenteral aspirin in large doses does, however, cause stomach damage, possibly because blood borne, highly acidic NSAIDs, accumulate in gastric mucosal cells.[54] Hence, two of the main theoretical objections to the importance of the "topical" action of NSAIDs in the pathogenesis of gastrointestinal damage have been answered in these studies.

The dual action of NSAIDs to uncouple oxidative phosphorylation–inhibit electron transport ("topical" action) and inhibit cyclooxygenase ("topical" and systemic action) provides a logical explanation for the mechanism and high prevalence of gastrointestinal toxicity of these drugs. Hence, uncoupling/inhibition following indomethacin (the precise site of absorption is in part determined by size, lipid solubility and charge of the NSAID, drug formulation, gastric pH, etc.) would result in diminished cellular ATP production, cellular calcium toxicity,[5] production of reactive oxygen species[6 55] resulting in increased mucosal permeability.[56 58] Increased intestinal permeability allows luminal aggressive factors access to the mucosa which results in an inflammatory reaction. The concomitant inhibition of cyclooxygenase (which occurs at picomolar concentrations of

NSAIDs and hence evident irrespective of the mode of administration of the drugs), with decreased prostaglandins, may then alter local blood flow and therefore be an important cofactor in driving the inflammation to ulcers.[1-6]

The importance of elucidating the mechanism of the "topical" damaging effect of NSAIDs lies in the fact that it may be possible to make them safer by chemical modification. Although NSAID damage to the gastrointestinal tract is clearly a multistage pathogenic process,[4] the results of this study suggests that uncoupling of oxidative phosphorylation or inhibition of electron transport by NSAIDs may be an early pathogenic event which underlies the "topical" phase of the biochemical damage. This suggests an alternative strategy for the development of "safer" NSAIDs, namely modifications of the carboxylic group common to most NSAIDs, to render the drugs ineffective as proton translocators.

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